ABSTRACT

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ISOLATION AND CHARACTERIZATION OF cDNAs DIFFERENTIALLY EXPRESSED BETWEEN $CD8^+$ AND $CD4^+$ CELL LINES

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To detect novel molecules involved in immune functions, a subtracted cDNA library between two closely related murine lymphoid cell lines was constructed using the suppression subtractive hybridization (SSH) technique. Both cells are cytotoxic T lymphocytes, however the 2C is a $CD8^+$ cytotoxic T cell line and the 5.9 is an inflammatory $CD4^+$ T cell line (Th1). They can both secrete the cytolysin or perforin and are significantly resistant to cell-mediated lysis. When these two cell lines were subjected to prolonged exposure to reagents that deplete cells of ATP (2-deoxyglucose, sodium azide, and potassium cyanide), the 5.9 cell line became substantially susceptible, but the 2C and other $CD8^+$ cell line still stood out as being strikingly resistant to granule-mediated lysis. A modified differential screening of colonies randomly picked from the

constructed subtractive cDNA library and RNA blot analysis were used to identify cDNA clones expressed in 2C and/or 5.9 cytotoxic T cell lines. Thirteen cDNA clones were isolated, from which seven were expressed in both cell lines, and only two were expressed in 2C but not in 5.9 cell line. All the isolated cDNA clones were sequenced. A search of the Gene Bank database with the BLAST X program revealed no extensive homology of 1B and 3F cDNA clones to known genes. Limited homology respectively to a zinc finger motif and the transcription factor MTF-1 were observed. Also RT-PCR analysis of cells purified from primary mixed lymphocyte reaction, demonstrated that both 1B and 3F cDNA clones were expressed only in $CD8^+$ but not in $CD4^+$ purified cells. This approach of employing subtraction coupled with partial cDNA sequence determination can be useful for a first selection of putative functionally relevant molecules.

ISOLATION AND CHARACTERIZATION OF cDNAs DIFFERENTIALLY EXPRESSED BETWEEN $CD8^+$ AND $CD4^+$ T CELL LINES

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CHAPTER I

INTRODUCTION

I.1 Cells and tissues of the immune system

I.1.1Cells of the immune system

Immature precursor cells differentiate into mature effector cells of the immune system. The precursor cells themselves are developed from the common progenitor cells known as pluripotent or common haemopoietic stem cell found in the bone marrow. These progenitor cells also proliferate and renew themselves and acquire new functional and phenotypic characteristics to become terminally differentiated such as erythrocyte, megakaryocyte, myeloid, or lymphoid progenitor cells. This process of blood cell production known as haematopoiesis is controlled by a variety of soluble secreted factors (cytokines/lymphokines) and hormones. At least eight distinct cells terminally differentiated arise as a result from a single hematopoietic stem cell.

a. The myeloid progenitor cell:

The polymorphonuclear (PMNs) leukocytes or granulocytes are derived from the common myeloid progenitor cell. The PMNs have a wide variety (poly) of differently shaped (morpho) nuclei and have granules in their cytoplasm. The PMNs comprise of

neutrophil, eosinophil, basophil, and mast cell. In addition, monocytes and macrophages also known as mononuclear phagocytes are derived from the common myeloid progenitor cell.

b. The Lymphoid Progenitor Cell

The lymphoid progenitor cell gives rise to lymphocytes. The lymphocytes, along with monocytes and macrophages are also known as mononuclear leukocytes (MNLs) even though lymphocytes derive from the lymphoid progenitor cells while the macrophages and monocytes derive from the myeloid progenitor cells (1). Only the lymphocytes have the ability to specifically recognize foreign antigen molecules such as those of microorganisms, a feature not possessed by any other cell. Upon stimulation by antigen, some lymphocytes are triggered to become effector cells performing the function to eliminate the immunogen, while others become long-lived memory cells that may persist for years and allow a more rapid response upon second exposure to the immunogen.

A third population of lymphocytes, known as Large Granular Lymphocytes, resembles lymphocytes, however they lack the major lymphocytes surface markers and have distinct granular cytoplasm. The progenitor of large granular lymphocytes has not yet been identified. Large granular lymphocytes include natural killer (NK) cells, killer (K) cells, and lymphokine- activated killer (LAK) cells (2). NK cells kill certain tumor cells and some virally infected cells, but do not recognize a specific antigen. K cells have molecules on their surface that act as receptors for the end of an antibody that does not bind antigen (the Fc region of the molecule). K cells express Fc receptors on their surface, through which they bind antibodies. Fc receptors allow K cells to focus

cytotoxic activity toward antibody coated-targets in a process referred to as antibody dependent cellular cytotoxicity (ADCC). The ADCC can also be utilized by some cytotoxic T cells as well LAK cells, like NK cells do not specifically recognize an antigen, but are capable of destroying a wider range of targets than NK cells. LAK cells may be derived from NK cells or cytotoxic T cells.

c. Dendritic cells

Dendritic cells belong to a group of specialized cells collectively called antigen presenting cell (APC) also called professional antigen presenting cell, that play a critical role in initiating immune responses. They may transport antigen within them or on their surface for presentation to naïve T cells and thus, like macrophages, play an important role in helping T cells to respond to antigen.

d. Megakaryocytes and platelets

Megakaryocytic progenitor cells are derived from haematopoietic stem cells. They undergo endomitosis (producing of multiple copies of DNA within the cell) and differentiate into megakaryocytes. Platelets are derived from megakaryocytes by a process, which appears to involve cytoplasmic fragmentation of the cell.

I.1.2 Tissues of the immune system

Lymphocytes are found circulating in the blood, but their large majority are found either in discrete cluster or organized in specific tissues. The components of this lymphoid system are categorized as primary, secondary or tertiary lymphoid tissue.

The development and differentiation of lymphocytes are done in the primary lymphoid tissues that include the thymus and the bursa equivalent tissues (which are the fetal liver and, probably, the adult bone marrow, respectively, in humans). They are responsible for

the production of mature "virgin " lymphocytes not yet exposed to antigen. Secondary lymphoid tissues allow the accumulation and presentation of antigen to both virgin and memory lymphocyte populations. The remainder of the body's tissues may be considered tertiary lymphoid tissues, where they normally contain only few lymphocytes, but, during an inflammatory reaction, may be invaded by unique subsets of memory lymphocytes. This dissertation will discuss mainly about the white blood cells, particularly the lymphocytes, because they mediate the antigen-specific adaptive immune response.

I.2 Lymphocytes

Lymphocytes are white blood cells that mediate the antigen-specific adaptive immune response. Two functional types of antigen-specific lymphocytes are distinguished: B lymphocytes develop into plasma cells that secrete antibodies and T lymphocytes develop into effector cells that kill other cells infected with intracellular pathogens or that activate other cells of the immune system including macrophages and B lymphocytes.

I.2.1 B lymphocytes

B cells comprise about 5-15 % of the circulating lymphocytes. A distinguishing feature of B cells is the expression of antibody molecules (immunoglobulin or Ig) on their surface, which act as the antigen receptor of the cell. The B cell antigen receptor consists of more than one group of molecules. In addition to the immunoglobulin molecule, the BCR comprises a duplex of molecules known as Igα and Igβ, which have a single extracellular domain and a cytoplasmic tail. Each antibody molecule contains a region,

which recognizes and binds to a particular antigen. Pre-B cells are derived from the common lymphoid progenitor. They are large and have parts of antibody molecules in their cytoplasm. They differentiate into immature and then mature B cells, which have antibody and other molecules on their membranes (CD19, CD20, CD23, CD24, CD35 and CD45). After antigenic stimulation, B cells proliferate and differentiate either into plasma cells, or small resting cells, which are able to respond to the next time that the same antigen is encountered (memory cells). Plasma cells are designed to produce vast quantities of antibody, which is secreted outside the cell.

I.2.2 T lymphocytes

Unlike B cells, T cells do not recognize normal antigen in its intact form, rather they detect antigen presented on cell surface as peptide fragments derived from the foreign proteins and bound to specialized cell-surface molecules. These specialized molecules encoded in a complex of genes were entitled major histocompatibility complex (MHC) because their potent effects on the immune response to transplanted tissues first identified them. These peptide-binding proteins are still called MHC molecules. The recognition of antigen as a small peptide fragment displayed by an MHC molecule at the cell surface is the most distinctive feature of antigen recognition by T cells. To be associated with MHC gene products, an antigen must be processed, because only short peptides may associate with the antigen-binding regions of these molecules.

I.2.3 Antigen Processing and Presentation

Two major pathways of antigen processing and presentation can be distinguished depending partly on sites of replication of infectious agents. Viruses and some bacteria replicate in the cytosol, while many important pathogenic bacteria and some eukaryotic

parasites replicate in the vesicular system that includes endosomes and lysosomes. This pathway also presents Neoantigens expressed by transformed cells. The function of CD8 T cells is to kill virus-infected cells. Pathogens and their toxin products in the vesicular compartments of cells are detected by CD4 T cell, a different class of T cells. Microbial antigens may enter the vesicular compartment in either of two ways. Some bacteria, such as the mycobacteria that cause tuberculosis and leprosy invade macrophages and flourish in cellular vesicles. Other bacteria in the extracellular spaces may secrete toxins and other products that are internalized by endocytosis. Here only B cells have a specialized means of specific antigen uptake in their surface immunoglobulin receptor. CD4 T cells that are specialized to activate other cells, fall into two functional classes: Th1 CD4 T cells that activate macrophages to kill their internal bacteria, and Th2 CD4 T cells, which activate B cells to make antibody. To produce an appropriate response to infectious agents, T cells must be able to accomplish the delivery of peptide fragments to the cell surface from the different intracellular compartments by two distinct classes of MHC molecule. MHC class I molecules deliver peptides generated in the cytosol to the cell surface, where the peptide: MHC complex is recognized by CD8 T cells. MHC class II molecules deliver peptides originating in the vesicular system to the cell surface, where they are recognized by CD4 T cell.

a. Generation of Peptides Presented by MCH Class I Molecules

In general, peptides associated with MHC class I molecules are processed in the cytoplasm in a highly important regulated mechanism by a large, ATP-dependent, proteolytic complex called the proteasome. This controlled mechanism allows only partial degradation and not complete reduction to constituent amino acids. In the

cytoplasm, the breakdown products are transferred to the lumen of the endoplasmic reticulum (ER) by the peptide transporters TAP1 and TAP2 (Transporter associated with Antigen Presentation). Once inside the ER, the peptides are to be modified further by local proteases, producing peptides of correct size for binding to MHC class I. Recent study has shown that only peptides 8-10 amino acids long can fit the binding groove of a class I molecule, this length being restricted by the interaction between the amino and carboxyl termini of the peptide and the extremities of the groove (3).

b. Assembly and Intracellular Transport of MHC Class I Molecules

The heavy and light chains of the MHC class I molecules are synthesized in the ER. At physiological temperature, the MHC class I heterodimer is unstable, but become stabilized when associated with presentable peptide. Other molecules transiently associated with class I heterodimer and released when peptides are bound are thought to help class I molecules achieve the correct folding upon synthesis. To be presented on the cell surface, MHC class I-bound peptide must be released from the ER. Upon release, the class I-peptide complexes are transported via the golgi apparatus and the trans-golgi reticulum to the cell surface. Antigen processing of MHC class I can be inhibited by two agents. Brefeldin A blocks movement proteins from the endoplasmic reticulum to the golgi apparatus, and the adenovirus E3/19K gene product specifically binds to MHC class I molecules and retains them in the ER.

c. Generation of Peptides Presented by MHC Class II Molecules

MHC class II gene products are expressed only on cells capable of endocytosis and present antigens derived from an extracellular source. Endosome and lysosome fusion after endocytosis and degradation of the antigen begins by initial destruction of the

pathogens. There are three classes of effector cells that can bind to antigens derived from different types of pathogens presented by the two different classes of MHC molecule.

Antigens derived from pathogens multiplying in the cytosol are carried to the cell surface by MHC class I molecules and presented to CD8 cytotoxic T cells that kill virus infected cells. Antigens derived from pathogens in the intracellular vesicles and those from ingested extracellular bacteria and toxins are carried to the cell surface by MHC class II molecules and presented to armed effector CD4 T cells that can differentiate into two types of effector T cells: the inflammatory CD4 T cells (Th1) that activate infected macrophages to destroy intracellular pathogens and helper T cells (Th2) that activate B cells to produce antibody.

I.3.1Mechanism of stimulation and activation of naïve T cells

The initial interaction of T cells with antigen-presenting cells (APCs) is mediated by cell adhesion molecules. Cell adhesion molecules are surface receptor molecules present on professional antigen presenting cells, which by virtue of their interaction with receptor molecules present on naïve T cells, insure that naïve T cells can adhere to APC to sense the presence of antigens collected by APCs. Antigen presenting cells, and particularly dendritic cells bind naïve T cells efficiently through interactions between lymphocyte function-associated antigens-1 (LFA-1) and CD2 on the T cell and intercellular adhesion molecules-1, -2, -3 (ICAM-1, -2, and-3) and LFA-3 on the antigen-presenting cell (**figure 1).**

Triggering of effector T cells requires not only stimulation of the antigen-specific receptors, but also either binding of class II or class I molecule respectively to CD4 or the

tertiary structure of a protein by the reduction of disulphide bonds, thus making it more accessible to other degradative enzymes that include the cathepsins B, D and E. Peptide fragments regardless their degree of degradation can bind to MHC class II, but required further processing by endosomal proteases to produce a minimal fragment, which protected from further degradation by the class II molecule itself.

d. Assembly and Intracellular Transport of MHC Class II Molecules

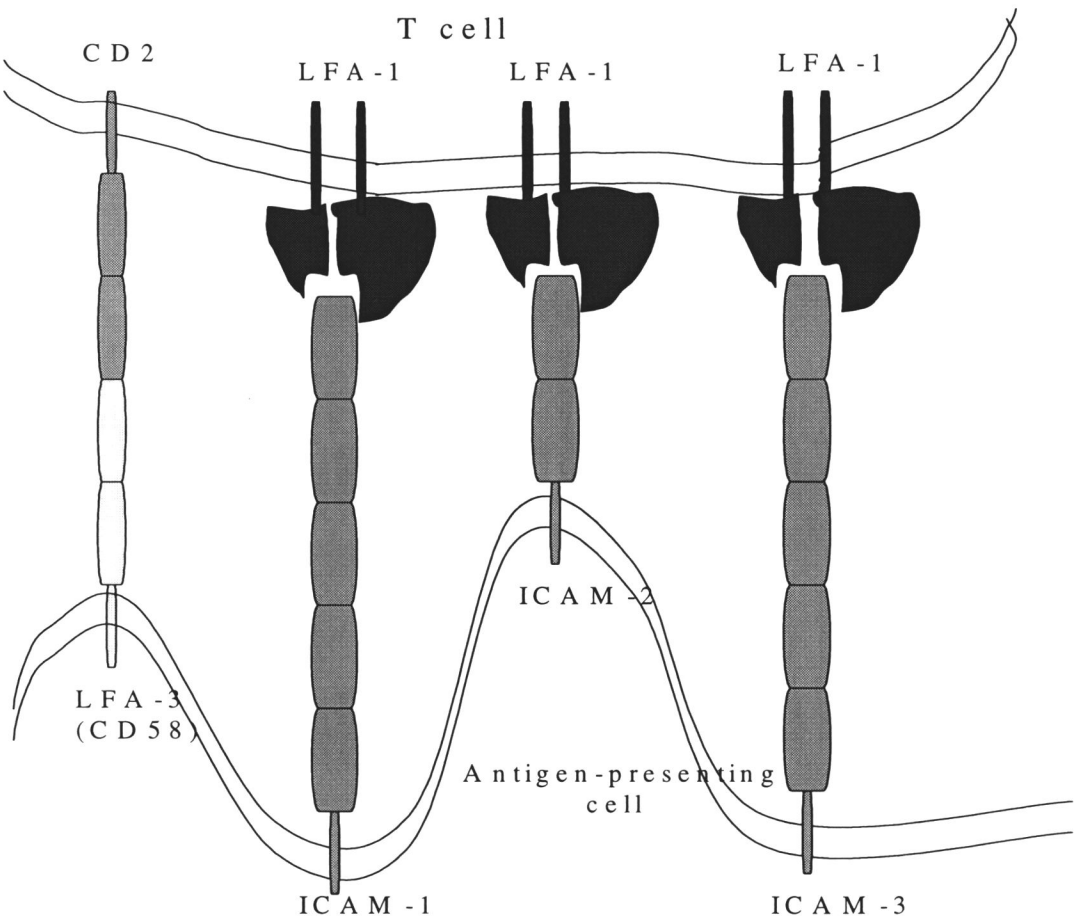
Structurally, class II and class I molecules are similar, but during the assembly, class II molecules are associated with a third molecule-the invariant chain. Thus, class II molecules are assembled in the ER as a trimeric complex (composed of an α and β chain which present the antigen at the cell surface and the invariant chain). In theory, Class I and class II molecules could bind the same peptides because both molecules are assembled in the ER. However, it had been shown by in vitro studies that class II molecules only bind peptide fragments after removal of the invariant chain, which usually occurs beyond the ER. This suggests that the invariant chain may help to regulate peptide binding, although presentation of cytosolic or ER-derived peptides may occur.

I.3 Specific cell-mediated cytotoxicity: mechanisms of cytotoxic T cells

T lymphocytes assume multiple roles in the defense of an organism against harmful infection. They can directly kill infected cells, activate macrophages to destroy intracellular pathogens or activate B cells to secrete antibody leading to destruction of extracellular pathogens. Adaptive immune responses are induced by antigen and gives rise to long-lasting protection against disease. Naïve T cells must be activated to proliferate and differentiate into armed effector cells capable of the removal of the

CD8 co-receptors bind to peptide: MHC complexes. A second co-stimulatory signal is required for clonal expansion of antigen-specific naïve T cells. For CD4 T cells, the co-stimulatory signal is delivered by B7, also known as BB1 molecule present on the same APCs on which the T cell recognizes its specific antigen. On T-cell, the receptor of B7 molecule is the molecule CD28. Once activated, the T cells express an additional receptor called CTLA-4 that binds B7 with a higher affinity than the CD28. In the absence of co-stimulation, antigen binding to the T cell receptor fails to activate cell and also leads to the nonresponsive state called anergy. The co-stimulatory signal is necessary for the synthesis and secretion of IL-2. The requirements for expression of IL-2 receptor are less stringent than those for IL-2 synthesis. As an example, only ligation of T-cell receptor is largely sufficient to induce expression of high-affinity IL-2 receptor on T cells. As discussed later, IL-2 made by one cell can act on IL-2 receptors expressed on neighboring antigen-specific cells. Naïve CD8 T cells require more co-stimulatory activity than naïve CD4 T cells, because their effectors actions are so destructive. This requirement can be met in two ways. First, activation on APCs with high intrinsic co-stimulatory activity can directly stimulate CD8 T cells to synthesize IL-2 that drives their own proliferation and differentiation. Second, to respond to some viruses and tissue grafts, cytotoxic T-cells require the presence of CD4 T cells during the priming of the naïve CD8 T cell.

Figure 1. Cell-surface molecules of the immunoglobulin superfamily are important in the interactions of lymphocytes with antigen-presenting cells. In the initial encounter of T cells with antigen-presenting cell, CD2 binding to LFA-3 on the antigen-presenting cell synergyzes with LFA-1 binding to ICAMs 1, 2, and 3 on the antigen presenting cell.


T cell
CD2
LFA-1
LFA-1
LFA-1
ICAM-2
LFA-3
(CD58)
Antigen-presenting
cell
ICAM-1
ICAM-3

In these responses, both naïve CD8 and CD4 T cells must recognize antigen on the surface of the same APC. It is thought that the action of the CD4 T cell in this case is necessary to compensate for co-stimulation by APC. This compensatory effect could occur in one of two ways. If the CD4 T cell is an armed effector cell, it may activate the APC to express higher levels of co-stimulatory activity, which would enable the APC to co-stimulate the CD8 T cell. Alternatively, the CD4 T cell may be a naïve or memory T cell secreting IL-2 in response to antigen and low levels of co-stimulatory molecules. IL-2 receptor being able to be induced by receptor ligation alone, the CD8 T cell may express IL-2 receptors even though it cannot produce the IL-2 it needs to drive its own proliferation. IL-2 in this case comes instead from an adjacent responding CD4 T cell. It is also known that addition of IL-2 can eliminate the need for a stimulatory signal for CD8 driven activation.

I.3.2Mechanism of cell mediated cytotoxicity

Cytotoxic lymphocytes that include CD4 CTLs, CD8 CTLs, NK cells, and LAK cells can kill virus-infected cells or tumor cells as already mentioned. Cells can die either by apoptosis or necrosis. Apoptosis, or programmed cell death, is a process by which cells activate, in response to external stimuli, a program leading within a few hours in typical morphologic alterations (membrane bebbling, chromatin condensation, cell shrinking, nucleus fragmentation). Ultimately, it results in death of the cells with formation of apoptotic bodies, which are normally cleared from the organism by professional phagocytes. Apoptosis differs from necrosis, uncontrolled and rapid cell death, by the fact that membrane integrity is normally preserved until the removal of the cells by phagocytosis, avoiding the risk for the dying cells to release their intracellular

content and to elicit inflammatory reactions. Cytotoxic T cells thus kill their target cells using at least three distinct mechanisms, all of which leading to cell death through the apoptotic pathway:

a. Secretion of cytotoxic cytokines

Most cytotoxic CD8 T cells release interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α), which contribute to host defense in several other ways. INF-γ has a direct inhibiting effect on viral replication and induces increased expression of MHC class I and peptide transporter molecules in infected cells, thus increasing the chance that infected cells will be recognized as target cells for cytotoxic attack. INF-γ also activates macrophages, recruiting them to sites of infection, both as effector cells and as antigen-presenting cells. TNF-α and TNF-β can synergize with INF-γ in killing some target cells through a cytokine-mediated pathway as well as through macrophage activation.

b. Calcium-independent contact-dependent cytotoxicity

In the absence of extracellular calcium, target cell death results from the interaction of the Fas ligand on the CTL (4) and the Fas receptor, a receptor of the TNF-receptor family (5), in the target cell membrane.

c. Calcium-dependent contact-dependent cytotoxicity

The calcium-dependent pathway relies on the secretion of cytotoxic granules onto the surface of the target cell and is, therefore, known as the granule exocytosis pathway. Perforin and granzymes are the two major molecules involved in the granule-mediated lysis. Both perforin and granzyme have been purified, cloned and characterized via loss-of-function models in transgenic animals.

I.3.3 Perforin and lymphocytes' granzymes

a. perforin characterization

Perforin, also called pore-forming protein (PFP), or cytolysin, or C9-related protein is responsible for the Ca^{+2}-dependent contact-dependent lytic activity of granules. It has been purified from murine, rat and human CTL, NK cell lines and stimulated peripheral blood lymphocytes (6, 7, 8, 9, 10). Perforin can be separated from other proteins after solubilization of granule proteins with high salt buffers by gel filtration chromatography (11, 12) or by affinity chromatography using an anti-C9 anti-serum. Perforin is a hydrophilic polypeptide with an apparent relative molecular weight (Mr) of 65-70 Kda under nonreducing condition in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS Page). When reduced, perforin results in a shift to 75 Kda, presumably due to disruption of internal cystein bonds. Perforin is found as a tubular polymer-polyperforin. Electron microscopy of target cell membranes lysed by purified perforin revealed the presence of typical tubular, ring-like polyperforin structures with inner diameter of 6-16 nm and height of 16 nm (13, 14). In the presence of Ca^{+2}, perforin undergoes ultrastructural changes involving a transition from its monomeric hydrophilic state into a polymeric amphiphilic form. The tubules formed by polyperforin contain approximately 12-18 molecules. Pores formed by perforin strikingly resemble lesions caused by the late events of complement activation, which comprise a sequence of polymerization reactions in which the terminal complement components interact to form the membrane attack complex (MAC), or the C9 component (15). The primary structures of murine, rat and human (16) perforin, have been independently deduced by cDNA cloning and sequencing. Alignment of the three predicted amino acid sequences reveals

considerable identity (69% of the residues are identical between mouse and human perforin).

The observations of common antigenic epitopes between the lytically active proteins of late components of the MAC and those of perforin had suggested that they were both derived from common ancestral genes (17, 18). Sequence comparison of amino acid between perforin and complement components reveals no significant overall sequence homology at the N- and C-termini (19) or with any other proteins in the databank. Approximately only 280 amino acid residues of the central segment of perforin were found to be homologous (20%) with a region of the complement proteins C6, C7 C8α, and C9 (20). This region of complements is believed to contain two functional important cysteine-rich regions: First, a putative lipid binding domain (LB), residues 167-211 in perforin, 292-333 in C9 is the most conserved region within the entire sequence and has been proposed to form two amphipathic α-helices that interact with the lipid bilayer. Second, the central segment, common to all complement proteins and perforin contains a cystein-rich domain of the low-density lipoprotein (LDL) receptor or epidermal growth factor precursor (21).

Despite their structural similarities, perforin and C9 complement interact differently with target cell membranes. Complement C9 insertion into large multilamellar vesicles or erythrocyte membranes is absolutely dependent on a receptor moiety assembled from the complement proteins C5b, C6, C7 and C8 (C5b-8) on the membrane (22). No receptor molecule has been yet reported for perforin. Perforin pore formation is organized into three main steps. Perforin binds to the target cell membrane, inserts into the lipid balayer and then polymerizes (23). Perforin binding to target cell is

strictly Ca^{+2} dependent, in contrast, to insertion that can proceed optimally at 37 °C in physiological ionic strength buffers. Controversy still exists whether perforin delivery to target cell by exocytosis is sufficient or even necessary for cell death. Purified perforin in contrast to whole granule preparations does not mediate DNA damage, suggesting that additional factors are needed. Since serine esterase inhibitors can inhibit DNA breakdown, several groups propose an involvement of granzymes in the induction of apoptosis (24). After DNA fragmentation during granule fractionation of either mouse CTL or rat natural killer cells, two groups had shown that granzyme A (25) and granzyme B (26) play a role in target cell DNA breakdown. In both cases, DNA breakdown is seen only if target cells are pretreated either with detergent or perforin. This result suggests that perforin is necessary for the uptake of the DNA degradation mediator into the target cell, but until now, the exact mechanism is remains unknown. Shi et al. (27) proposed that DNA-damaging molecule could be endocytosed by the target cells, as they attempt to repair the membrane damage induced by perforin. The DNA degradation mediator can also enter directly the target cells through the pores created by perforin.

b.Lymphocytes' granzymes

Granzymes are granule-associated serine hydrolases secreted by CTL and NK cells of murine, rat and human. Six highly related serine esterases, termed granzymes A-F, have been purified and characterized using granules extracted from murine- IL-2 dependent CTL clones (28); several have been shown to process proteolytic activity. In human, only four serine proteinases besides perforin and other undefined proteins were found in granules extracted from CTL and LAK cells. Two of the human granzymes are homologous to murine granzymes A and B. Granzyme A has a unique structure among

the serine proteases. It forms a disulfide-linked homodimer of 60 kDa, however granzymes B-G consist of a single polypeptide chain with relative molecular weight (Mr) ranging between 27 and 55 kDa. All granzymes, except granzyme C, are antigenetically related. They are highly homologous and all contain, at equivalent positions, the three residues His, Asp, and Ser, which form the catalytic center of serine proteases. Mouse granzymes B, C, E, and F have close physical link to the T-cell receptor-α (TCR-α)-chain locus on chromosome14 (29). In contrast, the mouse granzyme A gene is located on chromosome 13 (30). Human granzyme B, similarly is located close to the TCR-α/δ-chain locus on chromosome 14 (31). The gene for human granzyme A is localized on chromosome 5 (32).

Specific substrates are found only for granzymes A, B, and D. Granzymes A and D show trypsin-like activity cleaving best after Arg or Lys residues, although the activity of granzyme D is very low compared to that of granzyme A (33). Granzyme A cleaves Pro-Phe-Arg-7-amino-4-methyl-coumarin, Pro-Phe-Arg-nitroanilide, and BLT most efficient. Granzyme B has Aspase activity and efficiently hydrolyzes **Boc-Ala-Ala-Asp-SBzl**. Granzyme B also has significant activity towards **Boc-Ala-Ala-X-SBzl** substrates where X is Asn or Ser. The pH optimum of granzyme A and B for cleavage of the various substrates is around 8. Granzyme A is inhibited by PMSF, aprotinin DFP, and benzamidine. Granzyme B activity is blocked by isocoumarins. Using polymerase chain reaction (PCR) techniques, granzymes A-G are found to be expressed in long-term CD8 and CD4 T-lymphocytes. Specific species of mRNA for granzymes A and B and lesser extent for granzyme C were detected in both CD8 and CD4 T cells primed in vivo and in vitro short term culture of activated T cells. In contrast, no transcripts for granzymes D-

G were detected by PCR in either of these effector cell populations, indicating that these granzymes do not participate in T-cell-mediated function in vivo.

I.3.4 Apoptotic cell death pathway

The cell death that leads to apoptotic features is under genetic control. In mouse, a homolog of *ced-3* gene that was found to govern developmental cell death in nematode Caenorhaditis elegans (34-36) was found to be interleukin-1β (IL-1β) converting enzyme (ICE), a cysteine protease responsible for the processing of pro-IL-1β to the active cytokine (37). The finding that ICE overexpression in mammalian cells induced morphological changes associated with apoptosis (38) has led to discovery of several other family members of novel cysteine proteases that cleave after aspartic acid residues and can trigger apoptosis when activated (39-57) for this reason, a new designation of ICE-like proteases has been termed caspases, for cysteine aspartases (**listed in table 1**). Caspases exist in an inactive form in the cytosol of most cells as a single polypeptide chain that is activated by cleavage at specific Asp residues to form active heterodimeric protease. The observation that this cleavage can be mediated by caspases (either the same one or others) has led to the idea that apoptosis occurs as a consequence of a cascade of caspase activation. Consistent with this, in many cases, caspase function is required for cell death. As a classical example, in Caenorhabditis elegans, mutations in the CED-3 caspase can produce a phenotype in which there is no programmed cell death during development (40). In mammals, caspases are required in many situations for cell death. Peptide caspase inhibitors can effectively block apoptosis induced in cell lines by ligation of CD95, such that the cells remain fully viable (37). Based on these

observations, the caspases not only participate in the biochemical and morphological features of apoptosis but also determine whether or not the cell lives or die. Caspases that are activated as a consequence of cell membrane signaling events are classified as initiating or upstream caspases (caspases-2, -8 and –10). Inhibition of upstream caspasses prevents pro-apoptotic signal transduction via cytoplasmic receptors including the activation of acidic sphingomyelinase (47). The effectors, or downstream, caspases (caspases-3, -6, and –7) are activated later than the upstream caspases. These caspases are principally responsible for the cleavage of crucial substrates [PAK2 or DNA fragmentation factor (DFF) p45, also called inhibitor of caspase-activated DNase (ICAD)] involved in the apoptotic degradation phase. Downstream of caspase activation, the cleavage of caspase substrates leads to systematic dismantling of cell structures, which translates into apoptotic morphologic lesions (45). Upstream of caspase activation, a complex series of steps leads from extracellular death signals to caspase activation (44). First, extracellular ligands engage cytoplasmic membrane receptors. These receptors are part of, or activate transcription factors, which translocate into the nucleus. Second, these transcription factors induce the synthesis of molecules, which govern pathways leading to caspase activation and subsequent cell death.

Table 1. Nomenclature for interleukin-1β-converting enzyme (ICE/Ced-3 like human cysteine proteases (caspases).

Caspase designation	Alternative names	Refs
Caspase 1	ICE	51
Caspase 2	ICH-1	52
Caspase 3	Apopain, CPP32, Yama	53-55
Caspase 4	$ICE_{(rel)II}$, TX, ICH-2	56-58
Caspase 5	$ICE_{(rel)III}$, TY	56, 59
Caspase 6	Mch2	20
Caspase 7	Mch3, ICE-LAP3, CMH-1	23, 60, 61
Caspase 8	FLICE 1, MACH, Mch5	24-26
Caspase 9		27,62
Caspase 10	ICE-LAP 6, Mch6	28, 29
	FLICE 2, Mch4	

I.3.5 Simplified models of the perforin/granzyme and Fas mediated killing

The granules of CTLs and their constituent proteins are synthesized 24-48 hours after stimulation via the CTL receptor. The newly made perforin, granzymes and other constituent granule proteins are cytotoxic packaged in secretion granules (30). After the activated CTL recognizes its target, a tight junction is formed between the effector and the target cells and the CTL granules vectorially stream towards the site of contact (31) where the granules are fused with the effector cell plasma membrane and the granule contents are secreted directly to the target cell membrane. In the presence of calcium, perforin polymerizes and forms channels in the target cell membrane, through which the granzymes may pass. After entering the target cell, the granzymes are thought to pass into the cytoplasm of the cell, where they may act on specific substrates in the apoptotic cascade.

a. Mechanism of perforin/granzyme mediated killing

- **Probable perforin/granzyme mechanism leading to the target cell death**

Granzymes, particularly granzyme B, a serine esterase shows enzymatic specificity similar to that of some caspases (32). Accordingly, granzyme B is able to cleave and therefore activate caspase-3 (33) and a number of other caspases. It can also cleave some of the known caspase substrates (34). Based on these properties, granzyme B can trigger both caspase-dependent and caspase-independent signs of cell death (35-38). Granzyme B can, by itself cross the target cell membrane and move in a protected cytoplasmic compartment from which perforin might induce its release and thus allow its localization

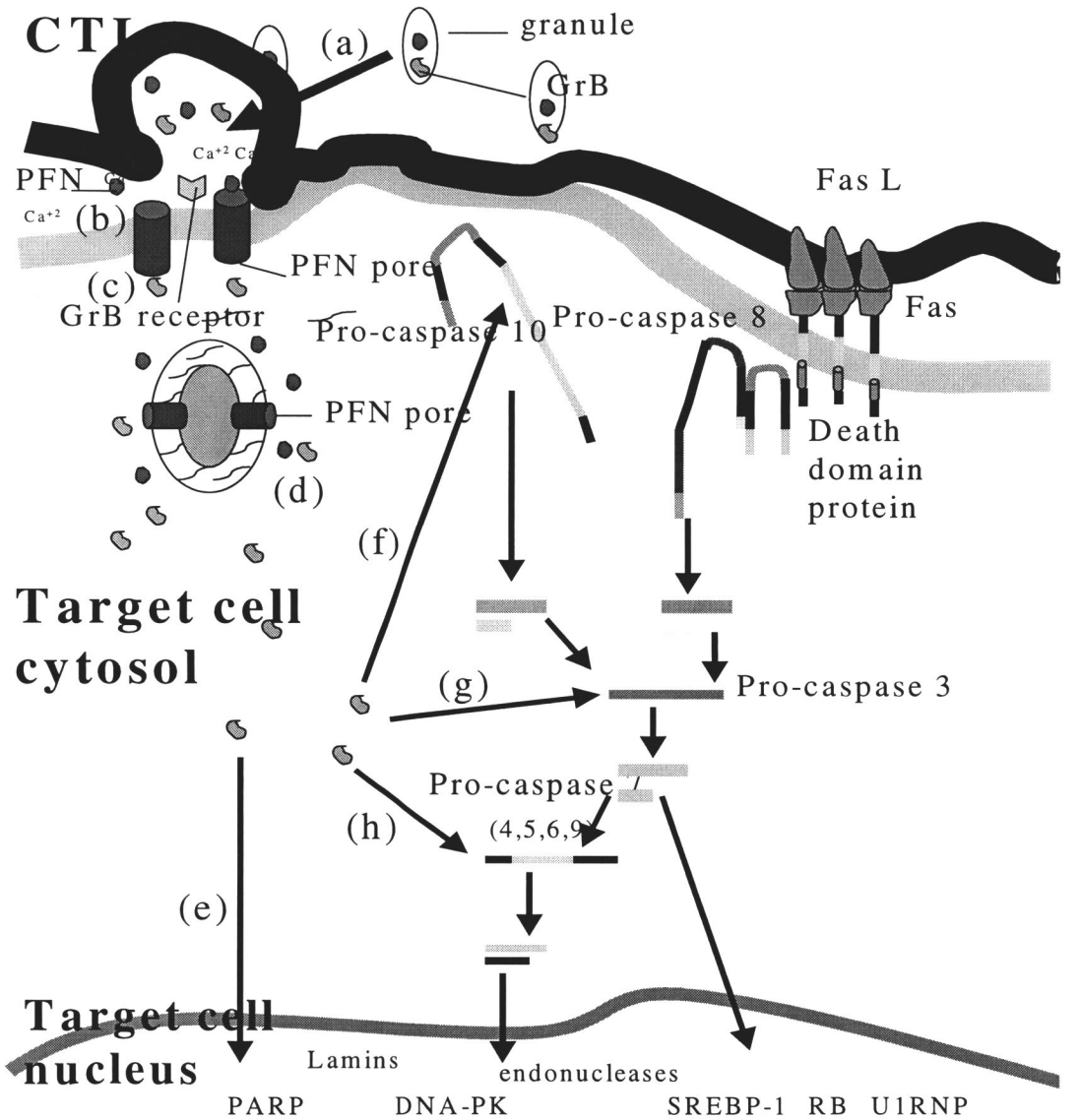
into the nucleus (39). It is not clear yet how, when, and where granzyme B acts on its various caspase and noncaspase substrates, leading to cell death.

- **Probable Fas pathway leading to target cell death**

One of the best understood mechanisms leading to target cell death is the Fas system. The Fas system employs the Fas receptor, a member of the TNF family of death receptor and the Fas ligand, a membrane-associated ligand that is synthesized within several hours after T cell receptor stimulation.

The display of the Fas ligand on T cell surface makes it possible for the Fas ligand to interact with Fas receptors on the surface of target cells. Engagement of the Fas receptor results in the aggregation of its intracellular death domains, leading to the recruitment of Fas-associated death domain (FADD) and procaspase-8 to form a death-inducing signaling complex (DISC). In the DISC, FADD and Fas interact via their death domain (40, 41); procaspase-8 then associates with FADD via the death effector domains of these two molecules (42-44). Procaspase-8, a member of the caspase family of cysteine proteases, is in turn activated by proteolytic cleavage after specific aspartic acid (Asp) residues. Activated caspase-8 is thought to activate additional downstream caspases by cleaving them after specific Asp residues, ultimately leading to the activation of procaspase-3 (45, 46). Activated caspase-3 cleaves an inhibitor molecule that is tightly associated with a nuclease known as caspase-associated Dnase (CAD) (47). Upon cleavage, this inhibitor of CAD (ICAD), also known as DNA fragmentation factor 45(DFF45) releases the nuclease from the CAD-ICAD complex, thereby allowing the nuclease to initiate DNA fragmentation and cause the ultimate fate of the cell: death.

Figure 2. A model of the mechanisms involved in perforin/granzyme and Fas mediated killing. After effector and target cells are conjugated, granules are (a) exocytosed into the space between the effector and the target cell. Perforin polymerizes in the presence of calcium, and may either (b) form pores in the target cell membrane through which (c) the granzymes pass. It is also thought that after interaction with specific binding sites, granzymes, specifically granzyme B is endocytosed during the cell membrane repair process. In this case, the granzymes are believed to be localized in a protected cytoplasmic compartment, from which (d) they can be released into the cytoplasm by the action of perforin. After release, granzymes can be transported directly to the nucleus (e) where they may act on death substrates, or they may act on death substrates within the cytoplasm (f, g, h). Granzyme B can cleave several procaspases to their active forms, leading to the activation of down stream caspases that are translocated to the nucleus where they can act on death substrates. The Fas system employs the Fas receptor on the target cell and the Fas ligand, synthesized after T cell stimulation. Engagement of the Fas receptor results in the aggregation of its intracellular death domains, leading to the recruitment of Fas-associated death domain (FADD) and procaspase-8 to form a death-inducing signaling complex (DISC). Activated caspase 8 lead to the activation of downstream caspases that also can act on death substrates in the nucleus leading to target cell death.


CTL
(a)
granule
GrB
Ca+2 Ca
PFN
Ca+2
(b)
(c)
GrB receptor
PFN pore
Pro-caspase 10
Pro-caspase 8
Fas L
Fas
PFN pore
(d)
Death
domain
protein
(f)
Target cell
cytosol
(g)
Pro-caspase 3
Pro-caspase 7
(h)
(4,5,6,9)
(e)
Target cell
nucleus
Lamins
endonucleases
PARP
DNA-PK
SREBP-1
RB
U1RNP

b. Resistance of cells to granule-mediated lysis

Cells resist perforin-mediated lysis by varying their degrees of sensitivity. This is done by the varieties of the repair processes developed by the cells, through which perforin pores are either removed from plasma membranes by endocytosis or ion gradients are restored by pumps (48, 49). Suppression of perforin channels by cellular ATP depleting reagents such azide and cyanide had shown that treated cells become increasingly susceptible for lysis, i.e. cells are even lysed at lower perforin concentrations. Cytotoxic T lymphocytes are known to be highly resistant to granule-mediated lysis (49, 50), or to lysis by intact CTLs *in vitro* (51, 52). When treated with ATP-depleting reagents, the CTLs remain resistant, in contrast to non-cytotoxic T cells (49). Experiments conducted on CTLs stimulated *in vivo* and let in culture for a very short period of time had shown resistance to granule-mediated lysis.

The resistance of CTLs to perforin-mediated lysis, not affected by ATP depletion suggests that CTLs' membranes are either resilient to integration of perforin or to the formation of pores. Studies using the Ca^{+2}-responsive fluorophore, indo-1 had demonstrated that no initial channels formation and then removal are observed in the CTLs when treated with granules (48). Susceptible tumor cells treated with sub-lytic concentrations of perforin show rise of intracellular free ca^{+2} concentration is followed by repair of membrane and then restoration of low intracellular free ca^{+2}. CTLs treated with 10 times the lytic dose for representative tumor cells manifest no initial rise of intracellular free ca^{+2}, suggesting that perforin lesions are prevented from forming in CTL membrane. It must be noted that CTLs, like other cells, are sensitive to other permeabilizing agents, such as complement or mellitin (49, 52).

CHAPTER II

RATIONALE

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are the immune system's first line of defense against cancerous and virally infected cells. Considerable evidence has shown that cytotoxic T Lymphocytes are stimulated to release toxic cytoplasmic granules when they adhere to and recognize surface antigens on target cells (53, 54). These granules contain, granzymes and a 70- Kda perforin, a pore-forming protein (55), also called cytolysin (56) that plays a key role in cytolysis mediated by CTL and NK cells (57-61). In the presence of Ca^{2+}, perforin binds to membranes and subsequently inserts into the lipid bilayer of the target cells to form oligomeric pores of 15 to 20 nm in diameter, resembling those of complement and some bacterial toxins. As a consequence, perforin lyses target cells. After a CTL has damaged its target cell, the same CTL can recycle to lyse new targets without going to self-destruction. In vitro studies show that cloned CTLs are highly resistant to lysis mediated by primary and cloned CTLs or isolated cytotoxic CTL granules (62-64). These observations suggest that CTLs possess a mechanism that protects them from their own lytic system. Furthermore, long-term, IL-2-driven CTL clones as well as primary CD8+ cells isolated from spleen (62-64) have been shown to be resistant to CTL-mediated lysis (65). Although the resistance of primary CD8+ T-cells is effective, it is not as pronounced as

the resistance displayed by the cloned CTL cell lines, suggesting that during long-term culture, there is some genetic selection for increased resistance to granule-mediated lysis How CTL acquires its resistance to granule-mediated lysis still remains unclear. Investigators have advanced two explanations.

First the resistance of cytotoxic T cell lines could be explained by the rapid and efficient removal of the inserted perforin from CTL membranes, reminiscent of many nucleated cells, which reduce their susceptibility to complement-mediated lysis (66). The second possible explanation of the resistance is that the T cell lines might have unusually active ion pumps that prevent or promptly restore the marked changes in intracellular ion concentrations, especially of Ca^{2+} (67), that result from insertion of perforin channels. These two possible mechanisms of resistance are well known to be cellular ATP-dependent. Verret et al. (49) demonstrated that exposure of prospective target cells to reagents (azide, cyanide, 2-deoxyglucose) that deplete cells of ATP, enhanced the susceptibility of most cells to granule-mediated lysis, with the exception of the $CD8^+$ CTLs that remain resistant. Based on this result, two modes of resistance to lysis were distinguished. An ATP dependent resistance (active), and the other being an ATP independent resistance (passive). The ATP-dependent mechanism may reflect the clearing of cell membranes of inserted perforin, similar to the mechanism utilized b many nucleated cells to reduce their susceptibility to lysis by activated complement through elimination of inserted complement attack complexes (C5b-8), possibly via endocytosis of affected plasma membrane (66). In addition, other investigators (Verret et al.,) found that the active resistance mechanism appears to be well developed in the $CD4^+$ T cells (both cytolytic and noncytolytic). The mechanism responsible for the ATP-independent

(passive) resistance of $CD8^+$ CTLs is still unknown. They also have shown that various tumor cell lines treated with various proteases such as trypsin, chymotrypsin, papain,or pronase became more sensitive to granule-mediated lysis. However, cytotoxic T lymphocyte cell lines, when treated in the same conditions still remained resistant. According to this result, it appears that the membrane component of CTL is either inaccessible to protease or not proteinaceous in nature. It is also possible that a protective molecule may be so abundant on CTL surface membrane that, it is not sufficiently destroyed by the conditions of proteolysis. Liu, c.c.et al. have found that CTL cell lines that had both de novo protein synthesis and newly synthesized RNA blocked with cycloheximide, emitine, or actinomycin D were still resistant to granule-mediated lysis; this implies that newly synthesized proteins are not required for the resistance of cytotoxic T lymphocytes. Based on these findings it has been hypothesized that a surface protein, or complex of proteins constitutively expressed on the membrane of activated or nonactivated CTLs, may be responsible of the ATP-independent resistance of cytotoxic T lymphocytes to their own pore forming protein.

The resistant state of a cytotoxic T lymphocyte (CTL) to their own pore forming protein would be controlled by the transcriptional activity of a set of genes. The assumption was made that these genes would not be expressed in other types of cells (tumor and non-cytotoxic cells). Molecules involve in the resistance of CTL to perforin might be found by looking for molecules specifically expressed in CTLs. In practice, a search for such molecules now often takes advantage of the power of molecular biology techniques, more precisely of the cloning and differentially screening of cDNA library, both techniques eventually optimized by subtractive enrichment.

The research project of my thesis dissertation focuses on finding molecules of mRNA transcripts differentially expressed in cytotoxic resistant T cells versus non resistant T cells, but using 2C and 5.9 cytotoxic T cell lines as model system. Although both cell lines are cytotoxic T cells and display resistance to granule-mediated lysis, only one, the 2C remains resistant in the ATP-independent manner.

To isolate and characterize mRNA transcripts differentially expressed, a subtracted cDNA library was first constructed between these two cell lines using the suppression subtractive hybridization technique (68). Randomly selected colonies from the library were differentially screened with the subtracted cDNA used as radiolabeled probe. The positively selected cDNA clones were confirmed by virtual northern hybridization. This is done by hybridizing each radiolabeled positively selected cDNA clones with the forward and reverse subtracted cDNAs blotted side by side on nylon membrane. Selected clones were sequenced and database search was conducted using the BLASTX sequence analysis program. Northern blot analysis of the selected clones revealed the cDNA clones that are truly differentially expressed (expressed on 2C but not on 5.9 cloned cell lines).

Cloned 2C and 5.9 cell lines were maintained in culture for several years with the support of high levels of IL-2, and repeated exposure to granules when periodically stimulated with target cells. These conditions may favor the induction of proteins. To find out whether the differentially expressed clones are not cDNA-induced by the culture conditions, their expression was examined by relative quantitative or standard RT-PCR in purified $CD8^+$ and $CD4^+$ T-cells form primary mixed lymphocyte reaction (MLR) using

gene specific primers. Cloning and functional characterization of each differentially expressed cDNA clone will be conducted as future experiments.

CHAPTER III

MATERIALS AND METHODS

In order to maintain ribonuclease-free conditions, special precautions were taken in the handling and preparation of solutions and glassware. All solutions except the Tris solution (Tris inactivates DEPC) and also all the microcentrifuge tubes were treated with DEPC (Diethylpyrocarbonate) overnight and then autoclaved before use in RNA and cDNA cloning. The glassware, particularly the electrophoresis tank used for electrophoresis of RNA was cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H_2O_2. After 10 minutes, at room temperature, the electrophoresis gel box was rinsed thoroughly with DEPC treated water.

III.1 Preparation of solutions

a. 5 M sodium Chloride (NaCl)

292.2 g of NaCl was dissolved in 800 ml of FS dd H_2O. The volume was adjusted to 1 liter with water and dispensed into aliquots and sterilized by autoclaving.

b. 1M Tris, pH 8.0

60.57 g of tris-base was dissolved in 400 ml of Fsdd H_2O. The solution was allowed to equilibrate with concentrated HCl to pH 8.0. FSdd H_2O was added up to 500 ml and then autoclaved. The solution was stored at room temperature

242 g of Tris-Base was dissolved into 800 ml of FS dd H_2O followed by the addition of 57.1 ml of glacial Acetic Acid and 100 ml of 0.5 M EDTA, pH 8.0. The volume was adjusted to 1 liter by the addition of water and the solution was stored at room temperature.

d. Ethidium Bromide (EtBr) (10 mg/ml)

0.5 g of ethidium bromide was added to a 50 ml falcon tube containing 50 ml of FS dd H_2O. The solution was stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The falcon tube was wrapped in aluminum foil and stored at room temperature.

e. DNA Sample Buffer 6X

0.25 % bromophenol blue

0.25 % xylene cyanol

30 % glycerol in water

Store at room temperature

f. 20% SDS

Solid sodium dodecyl sulfate (SDS) was dissolved at 68 °C for 45 minutes to make 20 % solution then cooled to room temperature before the pH was adjusted to 7.2. The solution was stored at room temperature.

g. 0.5M EDTA (Ethylenediamine Tetraacetic Acid)

To a bottle containing 73.05 g of EDTA, 300 ml of FSdd H_2O_2 was added. The pH was adjusted to 8.0 and then the volume was completed to 500 ml by addition of FSddH_2O_2. The solution was then autoclaved and then stored at room temperature.

h. 5M NaOH (Sodium Hydroxide)

To a bottle containing 40 g of NaOH, 200 ml of FSdd H_2O was added. The solution was autoclaved and stored at room temperature.

i. RNA Binding Buffer

0.5 M NaCl

10 mM Tris-HCl (pH 7.5)

1.0 MMEDTA

0.5% SDS

j. RNA Elution Buffer

10 mM Tris-HCl (pH 7.5)

1.0 mM EDTA

0.2% SDS

k. Guanidine Isothiocyanate (GIT) buffer:

To prepare 50 ml of GIT buffer, 23.63 g of guanidine isothiocyanate (4M) (Fisher Scientific) was dissolved in 49.165 ml of FSdd H_2O. A volume of 0.835 ml of sodium acetate, pH 6.0 (50 mM) was then added. The mixture was passed through a 0.45 μm filter, followed by the addition of 0.835 ml of 2-β-mercaptoethanol. The solution was stored in the dark at room temperature.

l. Cesium Chloride solution:

The solution was prepared by dissolving 24 g of CsCl in 25 ml of FSdd H_2O. A volume of 210 μl of 3M Sodium Acetate was added and then the solution was passed through a 0.2 μm filter. The solution was then stored at room temperature.

m. 2 X Proteinase K Buffer:

To a 50 ml conical tube containing 10 ml 1M of Tris, pH 7.5 (200mM), 2.5 ml of 0.5M EDTA, pH 8.0 (25mM), 3.75 ml of 4M NaCl (300 mM), 5.0 ml of 20% SDS and 537 µl of 18 mg/ml Proteinase K (200 µg/ml) were added. FSdd H_2O was then added up to 50 ml and then the solution was stored at –20 °C.

n. 20X SSC

To a bottle containing 800 ml of FS dd H2O DEPC treated, 175.3 g of NaCl and 88.2 g of Sodium Citrate were dissolved. The pH was adjusted to 7 with few drops of concentrated NaOH. The volume was completed to 1 liter with H2O and the solution was autoclaved and then stored at room temperature.

o. 10X MOPS [3-(N-morpholino)-propanesulfonic acid] running buffer

To a bottle containing 300 ml of FS dd H2O, 20.9 g MOPS was added. The pH was adjusted to 7 with NaOH followed by the addition of 8.6 ml of 3 M Sodium Acetate and 10 ml of 0.5 M EDTA, pH 8. The volume of the solution was brought to 500 ml by addition of FS dd H2O2. The MOPS solution was stored at room temperature in the dark.

p. 20X SSPE stock solution

To a bottle containing 800 ml of FS dd H2O2 DEPC treated, 175.3 g of NaCl, 27.6 g of NaH2PO4.H2O, and 7.4 of EDTA were dissolved. The pH was adjusted to 7.4 with NaOH and the volume completed to 1 liter. The stock solution was then autoclaved and stored at room temperature.

q. 50% Denhardt's solutions

This solution was prepared by mixing in a flask containing 300 ml of FS dd H_2O, 3g of ficoll 400, 3 g of polyvinyl pyrollidone and 3 g of BSA (Bovin Serum Albumin) (fraction V). This solution was stored in aliquots at –20 °C.

r. Salmon Sperm DNA (SSDNA)

In a 15 ml conical tube containing 50 mg SSDNA, 5 ml of FS dd H2O was added. The tube content was boiled at 95 °C for 10 minutes and then cooled at room temperature. The DNA was sheared by drawing several times through an 18gauge needle and then the volume completed with FSdd H_2O to 10 ml. The solution was stored in aliquots at –20 °C.

s. Sephadex G-50 column

Sephadex G-50 (medium) was added to distilled sterile water (10 g of dry powder yields 160 ml of slurry). The swollen resin was washed several times with distilled sterile water to remove soluble dextran, which can create problems by precipitating during ethanol precipitation. Finally the resin was equilibrated in water or TE (pH 7.6), autoclaved, and stored at 4 °C.

t. 20 RNA sample buffer

To a microcentrifuge tube containing 500 µl formamide, 169 µl of formaldehyde (37 % stock)), and 100 µl of 10 X MOPS buffer were added. The sample buffer was stored at 4 °C until needed.

u. Prehybridization/hybridization solution (30 ml)

To a 50 ml conical tube, 15 ml formamide, 3 ml 50x Denhardt's solution, 7.5 ml 20x SSPE, 750 µl 20 % SDS, and 100 µg/ml denatured Salmon sperm DNA were respectively added. For hybridization, 1 x 10^6 cpm/ml heat denatured probe was added.

III.2. Agarose gel electrophoresis

a. Preparation of standard agarose gel electrophoresis

Agarose gels were prepared by heating powdered agarose in 100 ml of 1 X TAE buffer to boiling in a microwave for 3 minutes. The liquid was allowed to cool to 60 °C and 5 µl of EtBr (10 mg/ml) were added. The gel was poured into an electrophoresis gel tray in which the well-forming comb was installed. After polymerization, the gel was immersed in 1% TAE buffer contained in the electrophoresis tank before the removal of the comb. Before loading the gel, 1/5 volume of 6 X loading buffer (6 X loading buffer was 25 % glycerol and 0.05% Bromophenol blue) was added to the DNA samples. 5-20 µg DNA/lane was loaded into each well and electrophoresed at 200 V for 1-2 hrs. After electrophoresis, the gels were visualized on a UV-light box and photographed.

b. Preparation of formaldehyde-agarose gel electrophoresis

1% denaturing agarose gels were prepared by heating powdered agarose in 75 ml of water to boiling in a microwave for 3 minutes. The liquid was allowed to cool to 60 °C and then 10 ml of 10x MOPS buffer, 17 ml of formaldehyde (37 % stock), and 5 µl EtBr (10 mg/ml stock) were respectively added. The mixture was then poured into an electrophoresis gel tray in a fume hood. While the gel is hardening, the RNA samples

were prepared adding in each corresponding tube, 15 µl sample buffer, 5 µl RNA (10-20 µg total RNA or 0.5-2 µg poly A^+ RNA) and 4 µl of FSdd H_2O. Each mixture was heated at 65 °C for 5 min and then quenched on ice. 4 µl of the loading solution (30% glycerol, dye) was added and the gel was loaded. The gel was run in a 1X MOPS buffer with recirculation overnight at 23 V. The gel was visualized on UV-light box to check the integrity of the RNA and then photographed.

III.3. Routine culture and plating of *E. coli*

a. Preparation of stock solutions of antibiotics

- **Kanamycin stock solution (Kan 25 mg/ml)**

125 mg of kanamycin was weighted out in a 15 ml conical tube followed by the addition of 5ml of water. The stock solution was aliquoted and stored at –20 °C. The final working concentration was 25 µg/ml.

- **Tetracycline stock solution (Tet 15 mg/ml)**

75 mg of tetracycline was weight out in a 15 ml conical tube and then 5 ml of water was added. The stock solution was aliquoted and then store at –20 °C. The final working concentration was 15 µg/ml.

- **Chloramphenical stock solution (Cam 34 mg/ml)**

170 mg of chloramphenical was dissolved in 5 ml of 100 % ethanol contained in 15 ml conical tube. The stock solution was then aliquoted and stored at –20 °C. The final working concentration was 34 µg/ml.

- **Ampicillin stock solution (Amp 100 mg/ml)**

To prepare a 100 mg/ml amp stock solution, 500 mg of ampicillin was dissolved in a 15 ml conical tube containing 5 ml of water. The stock solution was aliquoted and stored at –20 °C. The final working concentration was 100μg/ml.

b. Preparations of culture media and agar plates Luria Bernati (LB) broth

To a bottle containing 900 ml of water, 10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g of NaCl were added. The solutes were well dissolved by stirring and the pH adjusted to 7.0 with 5 N NaOH. The volume was brought up to 1000 ml by adding water. The solution was autoclaved and then stored at room temperature. For plates, 15 g/l of agar was added before autoclaving. When cooled to 60 °C, the appropriate antibiotic was added and plates were poured, and after solidification, they were stored at 4 °C until needed.

c. 2x YT medium

To a bottle containing 900 ml of water, 16 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl were added. The solutes were well dissolved and the pH adjusted to 7.0. The volume was brought up to 1000 ml, and the medium autoclaved and then stored at room temperature. For plates, 15 g/l of agar was added before autoclaving. When cooled to 60 °C, the appropriate antibiotic was added and plates were poured, and after solidification, they were stored at 4 °C until needed.

III.4. Cell cultures

a. Culture of 2C and 5.9 cell lines

The 2C and 5.9 cell lines were grown in K-10 medium (450 ml RPMI, 22.5 ml K supplement, 5 ml penn/strep, and 10% FCS), containing irradiated A20 cells plus

recombinant interleukin-2 (IL-2). For the 5.9 cell line, 50 μg/ml ovalbumine was added. After 3 to 4 weeks of culture, 6.0 x 10^7 and 4 x 10^8 cells were harvested respectively for 2C and 5.9 cell lines.

b. Preparation the mixed lymphocyte reactions (MLR)

Single cell suspensions were prepared in a petri dish by grinding between the frosted parts of two slides, the freshly removed spleen from the mouse (Balb/C or Black/6) followed by the addition of 10 ml of K10 medium. Each single cell suspension was then transferred into a 15 ml conical tube and then centrifuged at 1000 RPM for 5 minutes. The supernatant was discarded and then the cell pellet was resuspended into 3 ml of Tris-buffer ammonium chloride (NH_4Cl 0.15 M; $KHCO_3$ 1.0 M; NaEDTA 0.01M)

c. Generation of CD8 T cells by one-way MLR

To generate CD8+ cells, 3.4x10^6 Black/6 spleen cells were incubated with equal number, but irradiated Balb/c spleen cells. The flask was incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 5 days of culture, the cells were harvested and the CD8+ cells purified by negative selection.

d. Generation of $CD4^+$ cell ovalbumine primed

3.4x106 Balb/c spleen cells were mixed with 200 μg of ovalbumin and the mixture was incubated in a humidified atmosphere of 5% CO2 at 37°C. The cells were harvested after 5 days and the $CD4^+$ purified by negative selection.

e. StempSep For Murine Cells

StempSep is a negative selection system in which the unwanted cells are immunomagnetically labeled and bound to a magnetic column. The desired cells,

collected in the column flowthrough, have not had antibody bound to their surface therefore, they are suitable for further functional and phenotypic studies.

e.1. Sample Preparation

- **Recommended Media**

Phosphate Buffered Saline (PBS) modified with 2-6% Fetal Bovine Serum (FBS). PBS is a phosphate Buffer Saline without Ca^{2+} or Mg^{2+}. Each prepared MLR was resuspended at 2-8x10^7 cells/ml in PBS plus FBS plus 5% normal rat serum and incubated for 15 minutes at 4^{O}C in a refrigerator. Each cell suspension is now ready for antibody labeling.

e.1.1. Immunomagnetic labeling:

To each cell suspension, 10 µl antibody cocktail per ml of cell was added. The cells were well mixed and incubated at 4°C in a refrigerator for exactly 15 minutes. The cells were washed with PBS plus FBS prepared medium and 100 µl of anti-biotin tetrameric antibody complexes was added per ml of cells. The cells were well mixed and again incubated in at 4 °C for 15 minutes. To the labeled cells, 60 µl of magnetic colloid was added per ml of cells followed by a final incubation at 4 °C for 15 minutes. The cells at this step were now ready for magnetic separation.

e.1.2. Magnetic cell separation/ Procedure with gravity feed:

- **Column Assembly-Gravity Feed:**

The column was assembled as the manufacturer recommended. The magnet was placed in the stand and all the procedure was performed in a sterile environment. The stempsep column was removed from its sterile package and placed in the magnet without

touching the luer fitting. Be careful not to touch any surface with the tip of the luer lock otherwise, the magnet will grab the column. The 3-way stopcock was removed from its sterile package and aseptically attached to the column. The blunt end needle was removed from its sterile package, keeping the cover on the end of the needle until the column washing step of the column preparation. Aseptically, the hub was attached to the luer fitting on the stopcock, directly below the column, and all the connections were checked.

- **Column Preparation (0.3'' column)**

The plug was removed from the top of the column and the 3-way stopcock was set to allow flow from the side connection into the column. A sterile syringe (included in the kit) was filled with PBS (without FBS), the air bubbled were removed and then the syringe was attached to the side connection of the 3-way stopcock. Slowly, the plunger of the syringe was depressed to delivered PBS up the column until the level of PBS is above the stainless steel matrix of column. Placing a waste container below the blunt end needle washed the column, and the cover removed from the needle, the recommended medium (PBS plus FBS) was added to the top of the column. The 3-way stopcock was turned so the flow was from the column down through the needle. The recommended medium was added until three column volumes were collected. The column was kept wet at any time by stopping the flow when the fluid level was just above the column matrix.

- **Separation Procedure**

The immunomagnetically labeled sample was loaded on the top of the column. The stopcock was turned to start flow of media down through the needle into the collection tube. The sample was allowed to run into the matrix. The recommended medium was added to the top of the column until three column volumes plus the volume of the start sample were collected. This collected flowthrough fraction was the enriched cell fraction for the cell enrichment cocktails or the purged cell fraction for the purging cocktails. The remaining fluid was drained from the column into a waste container and the used column; syringe, stopcock, etc were disconnected and discarded into appropriate waste container. The enriched cell fraction was washed three times with K-10 medium and was now ready for FACS analysis.

d. FACS analysis of the purified $CD8^+$ and $CD4^+$ T cell populations

5 x 10^5 cells of each purified population (CD8 or CD4) were resuspended in 500 µl of K 10 medium and first, Fc block was added to a final concentration of up to 1µg/10^6 cells. Each mixture was incubated on ice for 10 minutes and then washed two times with K 10 medium. The cell pellet was resuspended again in 500 µl of K10 medium, followed by the addition of a mixture of R-PE anti mouse CD8a for the CD8 purified cells and FITC anti mouse CD4 for the CD4 purified cells. Each mixture was made so that R-PE anti mouse CD8a or FITC anti mouse CD4 had a final concentration of 1 µg/10^6 cells. The mixtures were incubated on ice for 30 minutes, washed 1 time with 1X PBS and then resuspended in 500 µl of 1X PBS. The mixture was washed two times with ice-cold PBS buffer, and then resuspended in 1 ml of 1X PBS. $CD8^+$ and/or$CD4^+$ cells were sorted from the resuspended cells by flow cytometry.

III.5. RNA preparations

a. Total RNA extraction from cultured cells and mouse tissues

Total RNA extraction from mouse tissues and cultured cells was carried out by two different methods. For preparation of large amount of total RNA from cultured cell lines, the Guanidinium-Cesium Chloride (69) method was used. Total RNA extraction from primary cultured cells and mouse tissues was performed using the Single-Step Guanidinium Acid-Phenol Method (70).

b. Large scale RNA preparation by Guanidinium-Cesium Chloride Method

The cultured cells were pelleted at 1000 RPM for 5 minutes in the Beckman centrifuge. The supernatant was poured off and the cell pellet washed two times with the K-10 medium. The cells were resuspended in18 ml of GIT buffer and vortexed vigorously. On the top of two SW41 centrifuge tubes containing each 3 ml of CsCl, 9 ml of the GIT-cell mixture was layered. The RNA was pelleted by centrifugation in the SW41 rotor for 21 hours at 32 K RPM, 20 °C. The temperature here is very critical because the CsCl precipitates at lower temperature. After the centrifugation, the GIT layer and part of the CsCl layer were carefully removed using a Pasteur pipette and house vacuum. The remaining CsCl layer was carefully decanted without disturbing the pellet still by using the Pasteur pipette. The RNA pellet appeared translucid. The RNA pellet was then rinsed with 1 ml of cold 80% EtOH (-20 °C) by swirling gently. The EtOH was carefully removed with a Pasteur pipette and the RNA pellet was air-dried. The pellet was then resuspended in 500µl of 1X PK buffer and transferred into a 1.5 ml of microcentrifuge tube followed by incubation at 50 °C for 60 minutes to completely solubilize the RNA. An equal volume of buffered phenol was added, mixed by gently

vortex and then centrifuge for 3 minutes at high speed to separate the phases. The aqueous phase was transferred to a new tube and re-extracted two times with phenol/chloroform, followed by a one time extraction with chloroform. To the aqueous phase transferred to a new tube, 1/10 volume of 3M sodium acetate, pH 6.0 and 2.5 volume of cold 100% EtOH were added. The mixture was inverted several times and left at –70 °C overnight. The tube was thawed and equilibrated at 40 °C for 10 minutes and the RNA was then pelleted by centrifugation at 13000 RPM for 45 minutes. The RNA pellet was washed in 1 ml 70% EtOH for 30 minutes and then in 100 % EtOH for 15 minutes and then air-dried for 10 minutes. The RNA pellet was resuspended in 500 µl of FSdd treated DEPC water quantitated by UV spectrophotometer reading of an aliquot at OD_{260} and then 20 µg was run on 1 % denaturing agarose gel.

c. Total RNA extraction from animal tissues

BALB/C and BLACK/6 mice were purchased from and housed at Morehouse School of Medicine. The mice were killed by cervical dislocation and freshly removed tissues, were quickly immersed in liquid nitrogen. The frozen tissues were wrapped in a small piece of aluminum foil and completely immersed in liquid nitrogen until needed. Each tissue was ground to a fine powder using a pestle and a mortar, keeping it frozen throughout by sequential addition of liquid nitrogen. To the powder, 800 µl of the denaturing solution was added drop wise, while homogenizing very well and then passed through a 21 gauge syringe several times to break chromosomal DNA. The solution was transferred onto a microcentrifuge tube and then 100 µl of 2 M sodium acetate pH 4 was added. The tube was inverted up and down manually for 10 seconds. A volume of 800

µl of phenol was added and mixed well and mixed well as described above. 200 µl of chloroform was added and also mixed well by inversion. The tube was incubated on ice for 15 minutes followed by a centrifugation at 13,000 RPM for 20 minutes using a bentch to microcentrifuge at room temperature. The supernatant was transferred onto a new tube followed by the addition of an equal volume of isopropanol (Sigma). The tube was then incubated at –70 °C for 10 minutes for precipitation and then the RNA precipitate was concentrated by centrifugation at 13,000 RPM for 30 minutes at room temperature. The supernatant was decanted and the RNA pellet was resuspended in 300 µl of the denaturing solution followed by another addition of 300 µl of isopropanol. The mixture was incubated again at –70 °C for 10 minutes. The RNA was spun down by centrifugation at 13, 000 RPM for 30 minutes at room temperature. The supernatant was discarded and the pellet resuspended into 70 % ethanol and left at room temperature for 15 minutes. The tube was centrifuged at 13, 000 RPM for 30 minutes and the supernatant was discarded. The pellet was washed with 100 % ethanol and then air-dried for 10 minutes. The dried RNA was resuspended in filtered sterile DEPC treated double distilled water and the concentration determined by OD spectrophotometric UV reading at 260. 20 µg of each total RNA was run on 1% formaldehyde denaturing agarose gel. An $OD_{260/280}$ ratio from 1.8 to 2.0 was considered as a good RNA preparation to be utilized in subsequent experiments.

For total RNA extraction from purified cells (CD8 and CD4 T cells), the same protocol is used, however the 800 µl of denaturing solution id added directly on the cell pellet.

d. Poly A^+ RNA purification from total RNA

Oligo (dT)-cellulose was resuspended overnight at room temperature in 1X binding buffer. The resuspended cellulose was then poured up to 1 ml mark into a sterile dispocolumn. The column was first washed with 30 volumes of the elution buffer and then equilibrated with an equal volume of 1X binding buffer. A solution of 1 mg/ml total RNA made in 1 mM EDTA (pH 7.0) was first heat-denatured at 70 °C for 5 minutes and then quickly chilled on ice. To the denatured total RNA, an equal volume of 2X RNA binding buffer was added at room temperature. The mixture RNA/2X RNA binding buffer was directly applied onto the column, and the eluate collected immediately in a sterile tube. As soon the entire mixture entered the column, one column volume of 1X RNA binding buffer was added onto the dispocolumn and the elution continued. The total collected eluate was again heat-denatured, and reapplied onto the column. This process was done for a total of two times and thereafter, the column was washed with 2 column volumes of 1X RNA binding buffer, collecting 1 ml of fraction at a time. The OD_{260} from each collected fraction was read. Initially, the OD_{260} of the collected fractions were high as the nonpolyadenylated RNA passes through the column. The later fractions had very little or no absorbance at 260 nm. PolyA^+ RNA was then eluted from the column with 3 column volumes of the sterile, RNase-free elution buffer, collecting 200 µl each of the eluted fraction. The OD_{260} of each collected fraction was read. The collected fractions of polyA^+ eluted RNA with absorbance at OD_{260} were pooled and 1/10 of the volume of 3M Sodium Acetate was added followed by the addition of 2.5 volume of 100% EtOH. The solution was mixed well and then stored at –20 °C overnight for precipitation. PolyA^+ RNA was recovered by centrifugation at 13,000 RPM for 30

minutes at room temperature using a bentch top microcentrifuge. The pellet was washed with 70% and 100 % EtOH and then air-dried as previously described for total RNA concentration. Each polyA$^+$ RNA was redissolved in 30 µl of FS dd DEPC treated water and spectrophotometrically quantitated. 2 µg of each purified mRNA was run on 1% formaldehyde denaturing agarose gel.

III.6. Genomic DNA preparation from mouse spleen

The freshly removed spleen was ground to a fine powder using a pestle and mortar, keeping it frozen in liquid nitrogen throughout. The ground tissue was resuspended in 40 ml of genomic DNA digestion buffer in which proteinase K was added to a final concentration of 0.1 mg/ml. The mixture was transferred into a 50 ml conical tube followed by an overnight incubation with shaking at 50 °C. The mixture was split into two conical tubes and to each tube, an equal volume of phenol and chloroform/isoamyl alcohol was added. The tubes were mixed very well by vortexing and then centrifuged for 10 minutes at 4000 RPM. The supernatants were transferred to new tubes and another phenol/chloroform purification was performed. To new tubes containing the supernatants, equal volume of chloroform was added followed by centrifugation at high speed. This was repeated and then the supernatants were transferred to new tubes, to which ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% EtOH were added. The content of each tube was gently mixed by inversion and the nucleic acid precipitate was visible at once.

The DNA precipitate was scooped out using the end of a glass rod and rinse in 70 % EtOH then in 100 % EtOH for few seconds. The precipitates were combined in a

microcentrifuge tube and air-dried. The DNA was allowed to redissolve overnight at 37 °C in 1 ml solution of 1 M Tris-Cl pH 8 and 0.5 M EDTA pH 8.The solution of nucleic acids was RNAse treated and then incubated overnight at 37 °C. After the overnight incubation, 40 µl of 5 M NaCl and 5 µl of 20% SDS were added respectively.

For the final purification, 10 µl 15.6 mg/ml of proteinase K was added to the mixture and incubated for 2 hours at 37 °C. Deproteination of the mixture was continued by addition of equal volume of phenol/chloroform and chloroform alone. The aqueous phase was transferred to a new tube, and two volumes of 100 % ethanol were added and the DNA precipitate was again scooped out. The nucleic acid was washed in 70% and 100% ethanol sequentially and then air-dried, before dissolving in 1 ml of FS ddH_2O and quantitated by spectrophotometry at OD 260 nm. A test gel was run using 10 µg of the prepared DNA.

III.7. PCR-Select cDNA subtraction

III.7.1 Oligonucleotides

1/ **cDNA synthesis primer**

5'-TTTTGTACAAGCTT$_{30}$-3'

2/ Adaptors:

Adaptor1

5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3

3'-GGCCCGTCCA-5'

Adaptor 2R

5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'

3'- GCCGGCTCCA-5'

PCR primers

PCR primer 1 5'-CTAATACGACTCACTATAGGGC-3'

Nested PCR primer 1 5'-TCGAGCGGCCGCCCGGGCAGGT-3'

Nested PCR primer 2 5'-AGCGTGGTCGCGGCCGAGGT-3'

III.7.2 First-strand cDNA synthesis

For the first-strand cDNA synthesis, each tube of tester and driver containing 2 μg of poly A+ RNA was spun to dryness using a speed vac concentrator and then the RNA was resupended into 4μl of FS dd H_2O. To each tube, 1 μl of cDNA synthesis primer (10 μM) was added to have a final volume of 5 μl. The mixtures were denatured at 70 °C in a thermal cycler for 2 minutes, and then quickly cooled on ice for 2 minutes. The tubes were briefly centrifuged and then, 2 μl of 5X first-strand buffer, 1μl of dNTP mix (10 mM each), 1 μl of diluted labeled FS dd H2O and 1 μl AMV reverse transcriptase (20 units/μl) were added. The tubes were again briefly centrifuged and immediately

incubated at 42 °C for 2 hours in an air incubator, after which transferring the tubes on ice terminated the first-strand cDNA synthesis reactions.

III.7.3 Second strand cDNA synthesis

Generation of the second strand cDNA was carried out by adding 48.4 µl of sterile H_2O, 1.6 µl of dNTP mix, and 4.0 µl of 20 X second-strand enzyme to each tube of first-strand cDNA. The contents were mixed and then briefly centrifuged. The tubes were incubated at 16 °C in a waterbath for 2 hours, followed by the addition of 2 µl of (6 units) T4 DNA polymerase. The tubes were mixed well and quickly spun down and transferred to a 16 °C waterbath and incubation continued for an additional 30 minutes. The second-strand cDNA synthesis reaction was terminated by the addition of 4 µl of 20 X EDTA/glycogen mix to each tube. The synthesized double-strand cDNAs were phenol/chloroform extracted once with method an equal volume of phenol and chloroform isoamyl alcohol (25:24:1). The aqueous phase was transferred to new tubes, containing an equal volume of chloroform: isoamyl alcohol (24:1). The tubes were vortexed and then centrifuged to insure complete removal of the organic solution from the aqueous phase. The supernatant of each tube was transferred to another new tube followed by addition of 40 µl of 4 M ammonium acetate and 300 µl of 100 % ethanol. The mixture was vortexed and then centrifuged at 14,000 RPM for 45 minutes. The double-strand cDNA pellets were washed with 70 % then 100% ethanol. The resulting pellets were then air-dried for 10 minutes. The double-strand cDNA tester and driver were resuspended into 100 µl of H_2O and passed through a G-50 sephadex spin column to remove the unincorporated nucleotides. Each purified reaction was concentrated in a

speed vac concentrator to dryness and then resuspended into 50 µl of FS dd H2O. For confirmation of the efficiency of the first-and second-strand cDNA synthesis, a test gel was run using 10 µl of each purified cDNA on 1% agarose gel which was dried on a 3 MM sheet and wrapped in commercial wrap (Saran). Autoradiography of the gel was performed by exposing on film at –70 °C overnight (Kodak XAR-5 film) with intensifying screen.

III.7.4 Rsa I digestion

A master mix of three restriction enzyme digestion reactions was made by combining 15 µl of 10 X Rsa I restriction buffer and 6 µl of Rsa I restriction enzyme. To each tube containing 43.5 µl of tester and driver cDNA, 6 µl of the master mix reaction was added. The tubes were vortexed, briefly centrifuged, and then incubated at 37 °C for 2 hours. To analyze the efficiency of the digest mixture, an aliquot of 5 µl was run on a 1% agarose gel and transferred onto a 3MM paper and an exposure on an X-ray film as described above. The remaining reaction was terminated by addition of 2.5 µl 20 X EDTA/glycogen followed by a phenol/chloroform extraction as described above. Each tester and driver sample pellet was dissolved in 5.5 µl of filtered sterile double distilled water. At this stage, the preparations of the driver cDNA were completed and stored at –20 °C until needed.

III.7.5 Adaptor ligation

A 1/5 dilution of the RsaI digested tester cDNA was made by addition of 8 µl of FS dd H2O to a tube containing 2 µl of the digested cDNA.

a. Preparation of adaptor-ligated tester cDNA:

A 12 µl of master mix reaction for the ligation reaction was prepared in a microcentrifuged tube by combining 6 µl of FS dd H_2O, 2µl of 5x ligation buffer, and 2 µl of T4 DNA ligase as described in Clontech protocol manual. Typically, the content the tube was as follow:

In the tube containing the population of tester1, 2 µl of the tester cDNA, 2 µl of adaptor-1, and 6 µl of the master mix reaction were respectively added. Similarly in the tube containing tester 2R, 2 µl of the tester, 2 µl of adaptor 2R, and 6 µl of the master mix were added. The tubes were briefly centrifuged, and incubated overnight at 16 ^{o}C in a waterbath. The reaction was terminated by the addition 2.5 µl of 20X EDTA/glycogen to each tube. The tubes were heated at 72 ^{o}C for 5 minutes to inactivate the ligase, briefly centrifuged and processed for ligation efficiency test.

b. Ligation efficiency test

To insure that at least 25 % of the cDNAs have adaptors on both ends, a ligation efficiency test was performed. The success of the experiment allowed the amplification of the fragments that spanned the adaptors/junctions of the tester-1 and tester-2R in the subsequent steps. The ligation efficiency test consisted of a 1/200 dilution of each adaptors ligated-tester cDNA. All the reagents were combined as described in Clontech protocol manual kit; an example of the experimental set up is described in the table below.

	1	2	3	4
Tester-I (µl)	1	1	0	0
Tester-2R (µl)	0	0	1	1
G3PDH3' primer (µl)	1	1	1	1
G3PDH5' primer (µl)	0	1	0	1
PCR primer 1 (µl)	1	0	1	0
Total volume (µl)	3	3	3	3

Briefly, a master mix for 5 reactions was made by mixing 12.5µl of 10 x PCR buffer containing 15 mM MgCl2, 2.5 µl of dNTP mixed 10 mM, 2.5 µl of high expand fidelity PCR system, and 92.5 µl of FS dd H_2O in a microfuge tube. To each experimental tube described above, 22 µl of the master mix was added. The tubes were vortexed, briefly centrifuged, and two drops of mineral oil were added.

The tubes were incubated at 75 °C for 5 minutes in a PCR thermal cycler (Perkin-Elmer 480 DNA thermal cycler) to extend the adaptors sequences. Immediately, the cycling was performed as recommended by Clontech protocol manual kit without modification. A total of 25 cycles were run, and then at the completion, 10 µl of each PCR product was analyzed on a 2 % agarose gel electrophoresis.

III.7.6 Sequential hybridization reactions

To enrich the cDNAs differentially expressed, two series of hybridizations reactions were performed.

a. First hybridization

4X hybridization buffer was left to equilibrate at room temperature for at least 20 minutes prior to initiating the hybridization experiment. If precipitate was not observed, the buffer was used; otherwise, the buffer is prewarmed at 37°C for 10 minutes. For the experimental 2C and controls provided by the manufacturer, the followings reagents were combined in two separate labeled hybridization tubes shown in the following table.

Component	Hybridization Sample I	Hybridization Sample II
Rsa I digested driver cDNA	2	2
Adaptor1-ligated tester1	1	0
Adaptor2R-ligated tester2	0	1
4 x Hybridization Buffer	1	1
Final volume	4	4

One drop of mineral oil was added to each tube, followed by a brief centrifugation. The samples were heat-denatured at 98 °C for 2 minutes, and an immediate incubation was done at 68 °C for 9 hours.

b. Second hybridization

To a tube containing 1μl of the driver cDNA, 1μl of 4 x hybridization buffer, and 2μl of FS ddH2O were added. The tube was well mixed and then centrifuged briefly. 1μl

of the mixture was transferred to a new tube followed by the addition of one dropped of mineral oil. The tube heat-denatured at 98 °C for 3 minutes using a thermal cycler. Simultaneously, the denatured driver cDNA was mixed with hybridization samples 1 and 2 following the steps described below.

A- Using a micropipettor, set at15 µl and fitted with a pipette tip.

B- The pipette tip was allowed to touch the mineral oil/sample interface of the tube containing hybridization sample 2.

C- The entire sample was carefully drawn halfway into the pipette tip with a small amount of mineral oil.

D- The pipette tip was removed from the tube, and drawing a small amount of air into the tip creating a slight air space below the droplet of sample.

E- The steps B through D were repeated with the tube containing the freshly denatured cDNA driver. The pipette tip contains now both samples separated by a small pocket of air.

F- The entire mixture was transferred to the tube containing hybridization sample 1, and pipetting up and down, followed by a brief centrifugation, mixed the content of the tube. The reaction was then incubated at 68 °C for 10 hours.

To the entire hybridization reaction, 200 µl of the dilution buffer was added and then mixed by pipetting up and down.

The mixture was immediately heated at 68 °C for 7 minutes and stored at –20 °C.

III.7.7 PCR amplification of the subtracted cDNAs

Differentially expressed cDNAs were selectively amplified. Prior to the amplifications, complementary strands of the adaptors were filled in by a brief preincubation at 75 °C, regenerating the template, the binding site for PCR primer 1. During the first amplification, only double strand cDNAs with double strand adaptor sequences on each end are amplified. In the second PCR, nested PCR primers are used to further reduce the background and enrich for differentially expressed sequences. The following describes the two PCR amplification experiments (primary and secondary PCRs).

a. Primary PCR

Three volumes of 1 X time PCR reactions was prepared by mixing in a microcentrifuge tube 7.5 µl of 10 x PCR buffer, 3µl of 10 µM PCR primer 1, 1.5 µl of High Expand Fidelity PCR system, and 55.5 µl of FS dd DEPC H_2O. The mixture was vortexed, briefly centrifuged, and then 24 µl was added to a tube containing 1 µl of the diluted secondary hybridization reaction. The content of each tube was mixed very well by pipetting up and down followed by the addition of one drop of mineral oil. A preincubation at 75 °C for 5 minutes was carried out and the cDNA templates were amplified as per manufacture protocol (Clontech).

b. Secondary PCR

A one third 1/3 dilution of the primary PCR product was made by the addition of 27 µl of FS dd DEPC H_2O into a microcentrifuge tube containing 3 µl of the PCR product. A master mix for reactions was made as described above with the exception that,

nested PCR primers 1 and 2 R were used. The mixture was vortexed, briefly centrifuged and then 24 µl of the master mix was added to a microcentrifuge tube containing 1 µl of the diluted primary PCR product. The content of the tube was mixed very well by pipetting up and down followed by the addition of one drop of mineral oil. The diluted primary PCR product was then amplified as recommended by the Clontech protocols.

At the completion of the PCR experiment, 10 µl of each PCR product was run on a 2 % agarose gel electrophoresis.

III.7.8 Subtracted cDNA library construction:

The PCR-select subtracted cDNA library was constructed by directly cloning the secondary PCR product in a T/A cloning vector (*Invitrogen*)

a. Recombinant subtracted cDNA construction using T/A cloning vector:

To a tube containing 2.5 µl of the secondary subtracted PCR product, 4 µl of the pCR-II T/A cloning vector (invitrogen), and 2 µl of the T4 DNA ligase and 9.5 µl of FS dd DEPC H_2O were sequentially added. The content of the tube was mixed very well and then briefly centrifuged. The ligation reaction was then incubated at 14 °C overnight.

a.1 Transformation of DH5α competent cells

- **Titration of the transformation reaction**

The frozen DH5α competent cells (GIBCO-BRL) were thawed on ice. Five borosilicate tubes were labeled and immediately placed on ice. The ligation reaction was diluted 5 times with FS dd H2O. To each labeled microcentrifuge tube, the ligation reaction was added as described below:

Tube #	**volume of ligation reaction**
1	1 µl of the stock solution
2	3 µl of the stock solution
3	2 µl of the diluted solution
4	4 µl of the diluted solution
5	6 µl of the diluted solution

50 µl of the competent cells was added to each tube and immediately placed on ice for 30 minutes. The tubes were transferred at 37 °C in a waterbath for 1 minute and then returned on ice for an additional 2 minutes. To each tube, 1 ml of 2xYT medium was added followed by incubation at 37 °C for 1 hour with shaking. Five hundred microliters of each transformation reaction were plated in duplicates in 2xYT agar plates containing 100 µg/ml of ampicillin. The plates were incubated at 37 °C overnight. Colony growth was observed in each plate and the dilution that yielded more colonies (>800) on plating was chosen for future transformation.

a.2 cDNA library construction

To obtain a cDNA library of 8,000 - 10,000 bacterial colonies, four tubes of the transformation were therefore carried out. After the incubation at 37 °C with shaking, the

content of the tubes was combined and then plated on a 150 mm petri dish of 2xYT agar containing 100 µg/ml of ampicillin. Plates were incubated overnight at 37 °C, a cDNA library of approximately 8, 000 bacterial colonies were obtained.

III.8 Screening of the subtracted cDNA library

To screen the subtracted cDNA, colonies randomly picked from the subtracted cDNA library were arrayed by cDNA dot blots on Immobilon –Ny^+ Transfer Membrane (MILLIPORE). Each bacterial colony randomly picked was grown overnight in 200 µl of LB-amp in a 96 well plate. Amplification of each cDNA insert was performed using a master mix of 100 x reaction yielding for 1 x reaction of 2.0 µl of 10 x PCR buffer, 0.6 µl of nested primer 1, and nested primer2R, 0.4µl of dNTP mix (10 mM), 0.2 µl of high fidelity PCR system enzyme, and 15.2 µl of water. Bacterial aliquots of 2 µl per well were transferred onto a new 96-well plate, to which 18 µl of the PCR mix reaction was added. The resulting mixture was amplified in a Perkin-Elmer GeneAmp PCR System 9700 as described in Clontech protocol manual kit. A gel electrophoresis of 1 % agarose was run by loading 12 µl of each PCR product.

To array the cDNAs on the nylon membrane, 5µl of each PCR product were combined with 5 µl of 0.6 N NaOH in a 96-well plate. The NaOH is used to denature the DNA for hybridization. 1.5 µl of each mixture was transferred onto the nylon membrane and this was done in duplicate in each square. Two identical blots were made: one used for the forward subtracted probe, and the other for the reverse subtracted probe. The two cDNA blots were neutralized for 4 minutes in 0.5 M Tris-HCl (pH 8.0) and washed with

FSddH$_2$O. The blots were backed for 1 hour in a vacuum oven at 80 °C, and then stored until needed.

III.8.1 Preparation of the forward-and reverse-subtracted cDNA probes

The forward-subtracted cDNA was made using the same sample stock of subtracted cDNA used to construct the subtracted library above. To make the reverse-subtracted cDNA, subtractive hybridization was performed with the original tester cDNA as a driver instead and, the driver was used as a tester. The primary PCR products from the PCR-select cDNA subtraction (forward and reverse) made as previously described, were amplified and the secondary PCR products were directly used as probes for differential screening. Prior to use any subtracted cDNA as a probe, the adaptor sequences were removed because despite their small sizes, these adaptor sequences may still have the potential to cause a very high background on an arrayed subtracted library.

a. Removal of the excess adaptor sequences

For the removal of the adaptor sequences, each secondary PCR product was phenol/chloroform extracted followed by ethanol precipitation as previously described and centrifugation. Each pellet was resuspended in 28 µl of water from which 3 µl was set a side for agarose gel analysis as control undigested. 3 µl of 10 X buffer and 1.5 µl of Rsa I restriction enzyme were added to each tube containing the PCR product. As additional control for efficiency of RsaI digestion, pUC 18 plasmid diluted to 25 µg/ml was used. The tubes were incubated at 37 °C for 2 hours. To each Rsa I digested PCR product, 1µl of Sma I restriction enzyme was added, followed by incubation at room temperature for 2 hours. A final digestion was conducted by the addition to each tube of

10 µl of 10 x restriction buffer, 1µl of restriction enzyme Eag I and 61 µl of FS dd water. The tubes were incubated at 37 °C for an additional 2 hours. Each digested PCR product was phenol/chloroform extracted and the supernatant was passed through a G-50 spin column to remove the digested adaptor sequences. Each purified PCR product was concentrated using the speed-vac concentrator to dryness and the pellet was resuspended into 50 µl of FSddH_2O. The final digested PCR products were analyzed on a 2% agarose gel; along side with a control non digested PCR product previously saved prior to the enzyme restriction PCR product experiments.

b. Labeling of cDNA probes by random priming technique

The cDNA probes were radioactively labeled using the high prime DNA labeling kit (Boehringer Mannheim). During this experiment, the subtracted forward, the subtracted reverse and the negative hybridization control cDNAs were labeled.

The tube containing each cDNA was heat-denatured at 95 °C for 10 minutes and quickly chilled on ice for 2 minutes. The tubes were rapidly centrifuged and then 3 µl of dNTPs (-dCTP) mix, 4 µl of High Prime reaction mixture, and 5 µl or 50 µCi [α-^{32}P] dCTP, 3000 Ci/mmol, aqueous solution were respectively added. The tubes were incubated at 37 °C for 1hour after which each reaction was terminated by the addition of 1 µl of 0.5 M EDTA. Filtered sterile DEPC treated water was added to a final volume of 100 µl. Each reaction was phenol/chloroform extracted and the resulting supernatant was passed through a G-50 spin column equilibrated with TE (10:1) to remove the unincorporated dNTPs. 2 µl aliquot of each purified labeled probe was saved for Cerenkov counted (10^6 CPM/ml) and the remaining stored until needed.

III.8.2 Identification of the differentially expressed cDNA clones

a. Differential screening of the subtractive sequences from the library

The forward and reverse subtracted labeled cDNA probes were hybridized to the forward subtracted clones arrayed on nylon membranes.

Each nylon membrane was placed into each hybridization screwcapped glass tube and 5 ml of the hybridization solution was added. The prehybridization reaction was performed at 42 °C for 5 hours with continuous rotation, after which the solutions were removed and discarded. Newly prepared hybridization solutions were added to the glass tubes with the corresponding probe (forward and reverse), which was heat-denatured for 10 minutes at 95 °C then chilled on ice. Each probe was carefully added to the corresponding tube at a final concentration of 10^6 CPM/ml of hybridization solution. The hybridization reaction was carried out at 42 °C with rotation for 18-24 hours. The next day, the hybridization reaction was carefully discarded in a ^{32}P waste container, and the filters were washed 3 times at low-stringency with the washing buffer 2xSSC/0.1% SDS at 42 °C for 30 minutes each time. Next, two high-stringency buffer (0.2xSSC/0.1%SDS) washes were performed at 65 °C for 15 minutes. Each nylon membrane was wrapped in commercial plastic wrap (Saran) and then exposed to Kodak-XAR film overnight between two intensifying screens at –70 °C.

b. Confirmation of the differentially screened cDNA clones

To confirm differential screening results, virtual northern blot was done by first running the forward-and reverse-subtracted cDNAs on agarose/EtBr stained gel electrophoresis. The gel was denatured by incubation in a denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 30 minutes. The solution was discarded and the gel was rinsed

briefly with FS dd DEPC treated water. The gel was immersed in neutralization solution (1.5 NaCl, 1M Tris·Cl, pH 8) for 30 minutes. The gel was rinsed in FS dd $H_2O$and blotted overnight onto Immobilon-Ny^+ transfer membrane (MILLIPORE). The membrane was baked at 80 °C in a vacuum incubator and stored wrapped in aluminum foil at –20 °C until needed.

Probe preparation was done starting with bacterial clones harboring differentially hybridizing cDNA sequences, which were grown for plasmids purification. Differentially expressed cDNA inserts were isolated by PCR using nested PCR primers 1 and 2R (Clontech). The PCR products were run on 1% low melting agarose gel electrophoresis and each PCR product was purified from the gel by electro-elution. The purified PCR product was ^{32}P-labeled as described for random prime labeling technique and hybridized with the membrane of virtual northern blot.

c. Partial sequencing of the selected cDNA clones

All confirmed differentially expressed cDNA clones by virtual northern hybridization were sequenced with SP6/T7 primers (*INVITROGEN*) using Dye or BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI 377 automated DNA sequencer according to the manufacturer recommendations. Approximately 400 bp were compared with databases

d. Pair wise comparison of the selected differentially expressed cDNA clones

To verify the absence of redundancy in the selected clones, all the positive clones were pair wise compared using BLAST sequence 2 programs (National Center for Biotechnology information).

d. Northern blot analysis

Total RNA was extracted from various types of cell lines as described before. 20μg of each total RNA was run on 1% denaturing agarose gel and transferred onto Immobilon-Ny^+ transfer membrane. Each radiolabeled probe corresponding to a positive cDNA clone on the virtual northern blot experiment was hybridized with the RNA membrane.

e. Southern blot with mouse genomic DNA

Mouse spleen DNA (5μg) was digested overnight with restriction enzyme (10 units, Promega). Following size fractionation on a 1% agarose gel at 100 V for 3 hours, the DNA was transferred to a nylon membrane. cDNA clones differentially expressed were ^{32}P-labelled as previously described. Filters were prehybridized at 42 °C for 4 hours followed by hybridization still at 42 °C overnight. The membranes were washed twice (15 minutes each time) in 2x SSC and 0.1% SDS at room temperature and twice in 0.1 x SSC and 0.1 % SDS at 42 °C. Following washing, filters were wrapped in Saran Wrap and exposed to film overnight.

f. **Expression of differentially expressed genes in primary lymphoid cells**

Total RNAs from limited number of cells (250,000 cells) purified from primary MLR by StempSep were used in RT-PCR. To each tube of purified $CD8^+$ and $CD4^+$ containing 10 μl of total RNA, 2 μl of random hexamer, 2 μl of dNTPs and 2 μl of FS dd H2O were added. The mixtures were denatured at 70 °C for 3 minutes, and chilled on ice

for 2 minutes. The tubes were briefly centrifuged and then, 2µl of 10 X RT-PCR Buffer, and 1 µl reverse transcriptase were added. The tubes were again briefly centrifuged and immediately incubated at 42 °C for 2 hours in an air incubator, after which transferring the tubes on ice terminated the first-strand cDNA synthesis reactions.

To quantitate and test the efficiency of the first-strand cDNAs synthesized, 18 S rRNA primers were used as internal control in RT-PCR reactions. These reactions were conducted by adding into each tube containing 5 µl of each reverse transcription reaction, 5 µl of 10 X PCR buffer, 1 µl of 18 S primers, 1 µl of dNTPs, 1 µl of Taq DNA polymerase, and 37 µl of FS dd H_2O. The amplification was performed as recommended by the Ambion protocol manual. For the expression of differentially expressed cDNAs, the amplification was done as described above, but using a set of gene specific primers, 1B#1 5'aaccctcccatacccat3' and 1B#2 5'cgacgcatctatcgg ag3' for 1B cDNA clone and 3F#1 5'cagagctgagtt ctc3' and 3F#2 5'aggaagagtcta atg3' for the 3F cDNA clone. The PCR products were run on 1% agarose gel electrophoresis and photographed.

III.9 Screening of λTriplEx cDNA library using as probes, the differentially expressed cDNA positive clones

III.9.1 Bacterial cultures plating

a. Primary Streak plates

5 µl of each frozen bacterial stock in 25 % glycerol (E. coli strains XL1-Blue and BM25.8) was streak on LB agar plate with antibiotic, lacking $MgSO_4$.

LB/tet plate was used for XL1-Blue and incubated at 37 °C overnight, while

LB/kan/cam plate was used for MB25.8 and incubated at 31°C overnight.

The two plates were wrapped sealed with parafilm and stored at 4°C until needed.

b. Working Stock plates

An isolated colony from each primary streak was picked and streak on LB/$MgSO_4$ with antibiotics. Each plate was incubated and the appropriate temperature corresponding to each bacterial strain as described above.

III.9.2 Titering the amplified library

An isolated colony was picked from the XL1-Blue working stock plate and inoculated into 20 ml of LB/MgSO4/maltose broth without antibiotics at 37 °C with shaking overnight. The bacterial cells were centrifuged at 5000 RPM for 5 minutes and resuspended in 7.5 ml of 10 mM $MgSO_4$. While four 90 mm plates were being warmed up at 37 °C for 15 minutes, all the dilutions were carried out as recommended by Clontech protocol. The four tubes were prepared and incubated at 37°C for 15 minutes as recommended the manufacturer protocol. To each tube, 5 ml of the melting LB/$MgSO_4$ soft agar was added. Each tube was poured rapidly onto LB/$MgSO_4$ agar plates. The plates were swirled to promote even agar distribution, and left to harden at room temperature for 10 minutes followed by incubation (inverted position) at 37 °C for 7 hours, after which the tubes were stored at 4 °C overnight. Well isolated plaques were observed in all experimental plates and none in control plate (no plaque). Plaque density correlated with decreased serial phage dilution and the plate with well isolated plaques was used for titer determination.

a. Hybridization Screening

The λ TriplEx library was screened using probes from positive clones identified during northern blot experiment. A library with at least 1x 10^6 independent clones is generally representative of mRNA complexity; therefore the initial screening of the true differentially expressed cDNAs was performed using 1x 10^6 phage plaques as the starting point.

b. **Generation of 10^6 independent plaques**

A one third dilution of the titer of the stock library lysate (3.2x 10^6 plaque-forming units/μl) was made serially by adding to a tube containing 2 μl of the stock library lysate, 58 μl of 1 X dilution buffer. Next, 20μl of the above dilution was added to 680 μl of 1 X dilution buffer in a new tube. 18 μl of the final dilution was used to obtain 50, 000 plaques/150 mm petri dish (20 plates).

c. **Plaque lift to nylon filters**

An isolated colony picked from the working stock of XL1-Blue host cells was inoculated into 15 ml of LB/MgSO4/maltose broth in a 50 ml falcon tube and incubated overnight at 37 °C with shaking. The cells were centrifuged for 5 minutes at 5,000 RPM, and the pellet resuspended in 7.5 ml of 10 mM $MgSO_4$. 18 μl of the above dilution of the library lysate was combined with 500 μl of the host culture. The bacterial culture and the phage mixture were incubated at 37 °C for 15 minutes. 9 ml of melting LB/$MgSO_4$ soft agar was added to the mixture and poured onto LB/$MgSO_4$ agar plates. The plates were left at room temperature for 10 minutes to allow the top agar to harden, before incubating at 37 °C for 9 hours. Plates were stored at 4 °C until needed.

Membrane filters were first numbered with a ballpoint pen for reference. The first filter was immersed in the DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes, followed by immersion in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8) for 5 minutes and finally in 2 X SSC for rinsing before blot-drying on 3 MM paper. The DNA was permanently fixed by baking the immobilon Ny^+ transfer membrane at 80 °C for 1 hour in a vacuum oven.

d. Hybridization using cDNA probes

Filters were processed prior to hybridization with labeled probes by pre-washing the filters as follow. The pre-wash solution was poured in a tray and then the baked filters were immersed in the solution and incubated with gentle rocking for 1 hour at 60 °C. This step reduced backgrounds. In a hybridization chamber container containing 25 ml of pre-hybridization solution, each pre-washed filter was stacked on the top of each other and completely immersed using forceps. The pre-hybridization was carried out from 2 hours to overnight at 42 °C with gentle rocking to insure constant wetting of all membrane filters.

The hybridization reaction was made by mixing in another (different) hybridization chamber, 10 ml of the hybridization solution and 100 µl of freshly denatured radiolabeled probe (10^9 CPM/ µg). The filters were transferred and stacked as described above. The hybridization was performed by incubation with gentle rocking overnight at 42 °C.

e. Washing off the probes from the filters

Excess probes were washed off the filters sequentially. First at lower stringency buffer (2 X SSC, 0.5 % SDS) at room temperature for 30 minutes (two times) then at

higher stringency buffer (0.2x SSC, 0.1x SDS) at 65 °C for 15 minutes (two times). The filters were wrapped in plastic commercial wrap (Saran) and exposed to film (KODACK X-RAY FILM) at –70 °C for 48 hours. After developing the film, the filters were aligned with the autoradiogram placed on fluorescent light box and the corresponding positive plaques were identified. An agar plug containing several plaques was removed and placed in 1 ml of sterile 1x lambda dilution buffer containing 5% chloroform. Positive plaques were replated at density 500-1000 plaques on a 150-mm plate. Single isolated plaques approaching 100 % hybridization were obtained after the third round of screening (tertiary screening).

f. Converting λ phages to plasmid recombinant

The conversion of a λTriplEx phage clone to a pTriplEx plasmid clone required excision and circularizing a complete plasmid from the recombinant phage. The plasmid is released by *cre*-recombinase-mediated recombination at *loxp* sites. Release occurred automatically when recombinant phage is transduced into bacterial host expressing *cre* recombinase. In this system, *E. coli* BM25.8 grown at 31 °C provides cre-recombinase activity. Here the conversion was performed on individual positive plaques picked from the tertiary screening plates. For the single plaque conversion, an isolated colony of BM25.8 host cells was picked from the working plate and immediately inoculated to 10 ml of LB broth in a 50-ml falcon tube. The cells were incubated at 31 °C overnight in a shaking incubator at 150 RPM. Using the overnight cell culture of BM25.8 host, 100 µl of 1 M $MgCl_2$ was added.

An agar plug of a well-isolated positive plaque from the tertiary screening was picked and transferred in ~ 500 µl of 1x lambda dilution buffer containing 5% chloroform. To elute the phage from the agar plug, the mixture was left at 4 °C overnight. In a 15 ml conical tube, 200 µl of the host cell culture and 150 µl of the eluted positive plaque were combined a new tube and immediately incubated at 31 °C for 30 minutes without shaking. After the addition of 400 µl of LB broth, incubation continued at 31 °C with shaking at 225 RPM for 1 hour. 50 µl of the infected cell suspension was plated on an LB plates containing ampicillin and incubated at 37 °C overnight. This gave well isolated colonies of recombinant bacteria plates. A single colony was picked from each clone and plasmid was prepared using NucleoBond Plasmid Kit (Clontech).

g. Size determination of the positive plaques

All the prepared plasmids were first run on a 1% agarose gel and those with larger size inserts were chosen for restriction enzyme digestion screening using EcoR*I*/Xba*I* restriction enzymes. Each tube contained 10 µl of each prepared plasmid; 40 µl of EcoRI master mix containing 2 µl of enzyme. The tubes were incubated at 37 °C overnight. To each digested reaction, water was added up to 100 µl followed by ethanol precipitation and centrifugation. The resulting pellet was resuspended in 10 µl of water and 40 µl of the Xba*I* master mixed containing 2 µl of the enzyme, before incubating overnight at 37 °C. Each double digested reaction was ethanol precipitated, and centrifuged in eppendorf microcentrifuge. The pellet was resuspended in 10 µl DNA 1 X loading buffer before electrophoresis on 1% agarose gel.

CHAPTER IV

RESULTS

Previous studies have shown that when both the $CD8^+$ CTL cell line and the $CD4^+$ CTL cell line were exposed to reagents that deplete cells of ATP, only the $CD8^+$ CTLs remained passively resistant to granule-mediated lysis. As an initial investigation toward identifying gene (s) that are present in $CD8^+$ CTLs but absent in $CD4^+$ CTLs, gene (s) that may afford the resistance advantage to lysis of $CD8^+$ CTLs, this researcher sought to isolate and characterized the molecules that are specifically expressed in CD8+ cell lines. This chapter of the thesis is divided into four sections. Each of these sections incorporates at lest one major aspect of my thesis research.

In the first section (section A) is described the principle of the technique used to construct the subtractive cDNA library.

In the next section, the initial series of experiments reported in section B, describes the preliminary experiments prior the construction of the subtracted cDNA library.

In section C are described the experiments used to isolate and characterize the differentially expressed cDNAs.

In the final section (section D), the expression of the isolated differentially expressed cDNAs was first determined in primary purified lymphoid cells by RT-PCR, and then in attempt to recover full-length cDNA sequences, the differentially expressed

IV.1 Principle of the suppression subtractive hybridization technique

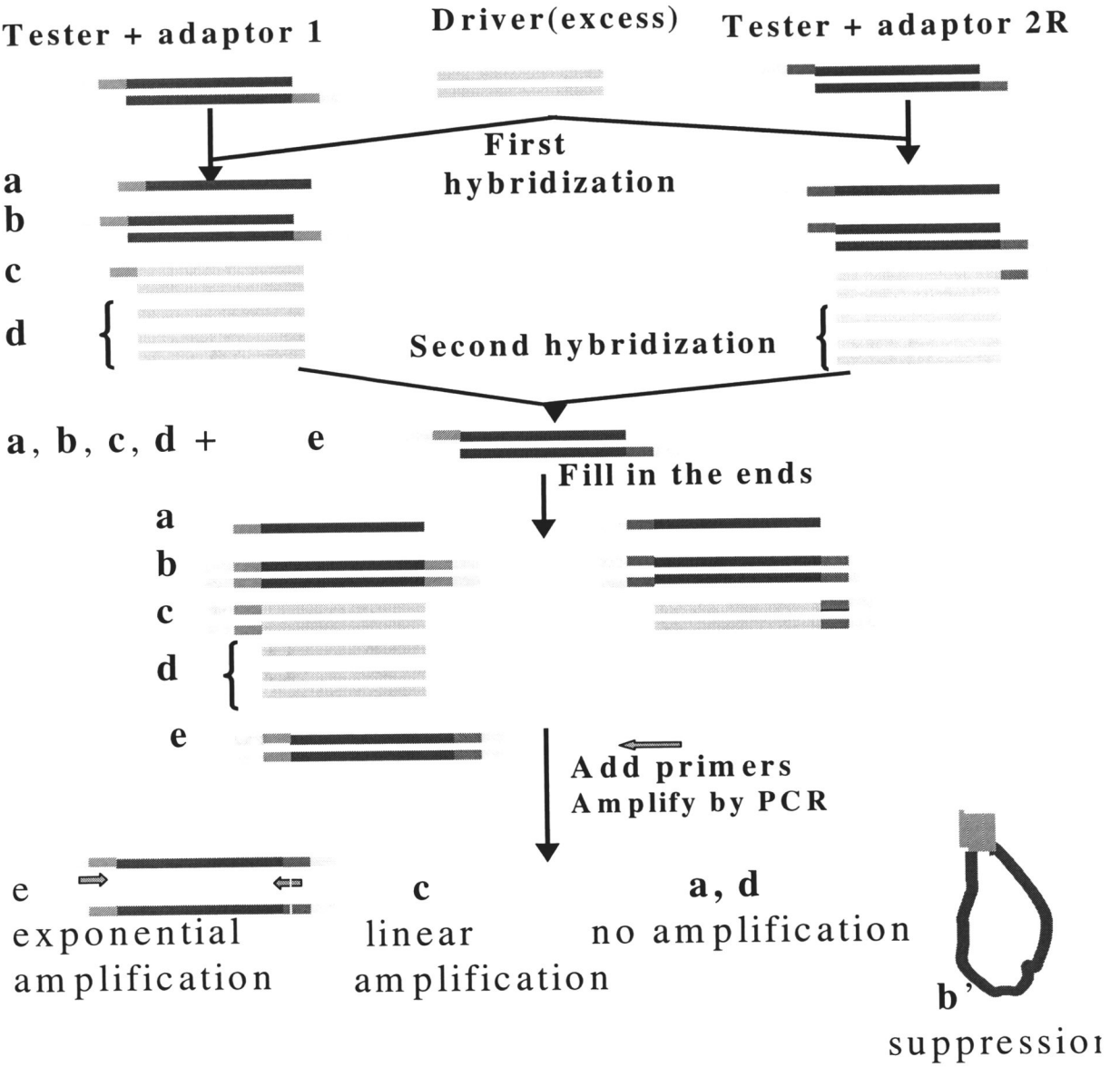
A schematic representation of the SSH method is shown in figure 3. The suppression subtractive hybridization (SSH) was performed between 2C (tester) and 5.9 (driver) cytotoxic T cell lines to isolate cDNAs or mRNAs differentially expressed. The 2C cell line is a CD8 cytotoxic T cell that kills virus infected target cells, and the 5.9 is an inflammatory CD4 T cell that activates macrophages to kill the pathogens they harbor. Thus the tester cDNAs contain the target genes specifically expressed, and the driver cDNAs are used for control comparison, since they are deprived of target molecules. Double-stranded (ds) cDNA was prepared from the tester and driver mRNA. In this process, tester and driver cDNAs are each digested with the fragment cutter enzyme RsaI, a restriction enzyme that yields blunt ends cDNA and short fragments. The resulting tester cDNA fragments are then subdivided into two populations (referred in this thesis as I and II) and each is ligated with a different double-stranded cDNA adapters referred as I and 2R at their 5'ends in separate tubes. Two hybridization stages are used during this subtraction technique. In the first stage, an excess of driver cDNA is added to each sample of tester cDNA. The samples are then heat-denatured and allowed to anneal, generating the type a, b, c, and d molecules in each sample (figure 3). During the hybridization, tester molecules that have a complement in the driver (redundant molecules) mostly form driver-tester heterohybrids and are removed from the single-stranded fraction. This process is much more effective for the more abundant redundant molecules due to the second-order kinetics of hybridization, leading to the approximate equalization of concentrations of various types of single-stranded redundant molecules. The target differentially expressed molecules do not interact with driver, and therefore

single-stranded tester becomes target enriched. Target molecules can still reassociate. Again, reassociation is much more observed for high-abundance target molecules than the low-abundance ones.

During the second stage, the two samples are mixed together without denaturing. The remaining single-stranded molecules are therefore given another chance to reassociate, forming double-stranded molecules. Redundant single-stranded molecules of tester will primarily form hetero-hybrids with their driver complements, whereas the single-stranded target molecules differentially expressed and enriched have no chance to become double-stranded other than to anneal to their corresponding antisense strand from the tester generating a new type e molecule. These new hybrids are double-stranded tester molecules with two different ends, which correspond to the sequences of adaptors 2R and I. A freshly denatured driver cDNA is again added to further enrich fraction e for differentially expressed sequences. After filling in the ends by DNA polymerase, the type e molecules, the differentially expressed tester sequences have different annealing sites for the nested primers on their 5' and 3' ends. The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences. During PCR, type a, and d molecules are missing primer-annealing sites, and thus cannot be amplified. Due to the suppression PCR effect, most type b molecules form a pan-like structure that prevents their exponential amplification. Type c molecules have only one primer-annealing site and can only be amplified linearly. Only type e molecules, which have two different adaptors, can be amplified exponentially. These are the equalized, differentially expressed sequences.

A secondary PCR amplification is performed using nested PCR primers to further reduce any background PCR products and enrich for differentially sequences. The subtracted cDNA was then inserted into a cloning vector. The subtractive hybridization was performed using the Clontech PCR-Select subtraction Kit (Clontech Laboratories Inc., Palo Alto, USA). In all technical details, the protocols described by the manufacturer were followed. All PCR and hybridization steps were performed on a Perkin Elmer 480 thermal cycler.

Figure 3. Molecular basis of the PCR-Select cDNA subtraction



Molecular basis of PCR-Select cDNA subtraction

IV.2 Construction of the subtracted cDNA library

To construct the subtracted cDNA library, total RNAs were first extracted from 2C, 5.9 and other cell lines (B2DIII, 5.5, EL4, A20, Yac-1 and 3T3 cell lines), and then fractionated by 1% formaldehyde agarose gel electrophoresis to check the integrity of total RNAs. As shown in figure 4, two strong ribosomal RNA bands running 28 and 18 S are observed, demonstrating that the RNAs are not degraded. Poly (A^+) RNAs were purified from total RNAs extracted from 2C and 5.9 cell lines by two cycles of oligo (dT)- cellulose chromatography (see material and methods). The first-and second strand cDNA of the tester and driver were synthesized, and to confirm their efficiency, a test gel was run, dried on a 3 MM Whatman paper sheet and then wrapped in a commercial wrap. Autoradiography of the gel was performed by exposing on film (Kodak XAR-5 film) overnight with intensifying screen. Figure 5 showing a pattern of smear between 0.5 and 12 kb in each lane demonstrates the synthesis of double strand cDNAs. The synthesized tester and driver ds cDNAs were digested with RSA I restriction enzyme to yield short and blunt-ended cDNA fragments. Uncut ds cDNA (tester, driver, and controls), and RsaI digested ds cDNA (tester, driver, and controls) were run on a test gel. The gel was dried on a 3 MM Whatman paper sheet, wrapped in Saran wrap and exposed on a film overnight with intensifying screen to check the efficiency of RsaI restriction enzyme digestion. Figure 6 shows that after the RsaI digestion, the average cDNA size is decreased. At this stage, the preparation of the drivers' cDNA was completed and stored at –20 ^{o}C until needed, but the resulting tester cDNA fragments were subdivided into two samples and each sample of tester cDNA was ligated at their 5' end to different double stranded adaptor I or 2R cDNA in two different tubes. To insure the success of ligation

reactions, PCR reactions using primer1, specific to the two adaptors and 3' or 5' GAPDH specific primers were used to carry out the ligation efficient test. Figure 7 shows a pattern of smear seen on lanes 2 and 4 indicative of amplified fragments between the two adaptors and 3' or 5' GAPDH primers, on lanes 3 and 5 a specific band of 0.5 kb is seen corresponding the predicted fragment between 3'and 5'GAPDH primers. These results demonstrate that the ligation reaction was successful. Sequential hybridization reactions were therefore proceeded. An excess of driver cDNA was added to the tubes containing each adaptor ligated tester cDNA to perform the first hybridization reaction. The samples were then heat-denatured and allowed to anneal. For the second hybridization, the two samples from the first hybridization were mixed together and a freshly denatured driver cDNA was added to further enrich for differentially expressed sequences. Prior to thermal cycling, the missing strands of the adaptors were filled in by a brief preincubation at 75 ^{o}C to create the binding site for PCR primer 1. In the first amplification, only double strand cDNAs with different adaptor sequences on each end was exponentially amplified. In the second amplification, nested PCR is used to further reduce the background and enrich for differentially expressed sequences. The secondary PCR product was directly inserted in a T/A cloning vector, and the ligation reaction was used to transform bacterial DH5α competent cells to obtain the subtracted cDNA library. Figure 8 shows the concepts behind the T/A cloning method.

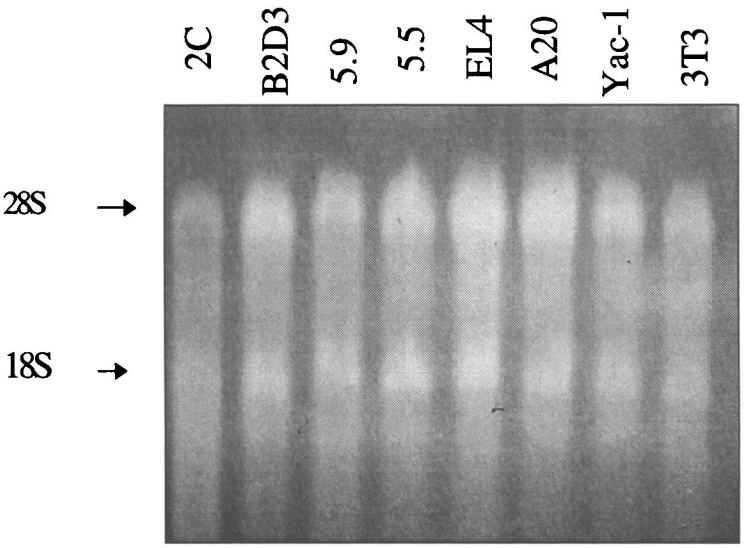


Figure 4. Electrophoretic pattern of ethidium bromide stained RNA. Total RNA (20µg/lane) was electrophoresed through a 1% formaldehyde-agarose gel containing 0.5 µg/ml ethidium bromide at 23 V overnight. Seen here are the strong ribosomal RNA bands running at 28 and 18S respectively, Demonstrating t that the RNAs are still intact.

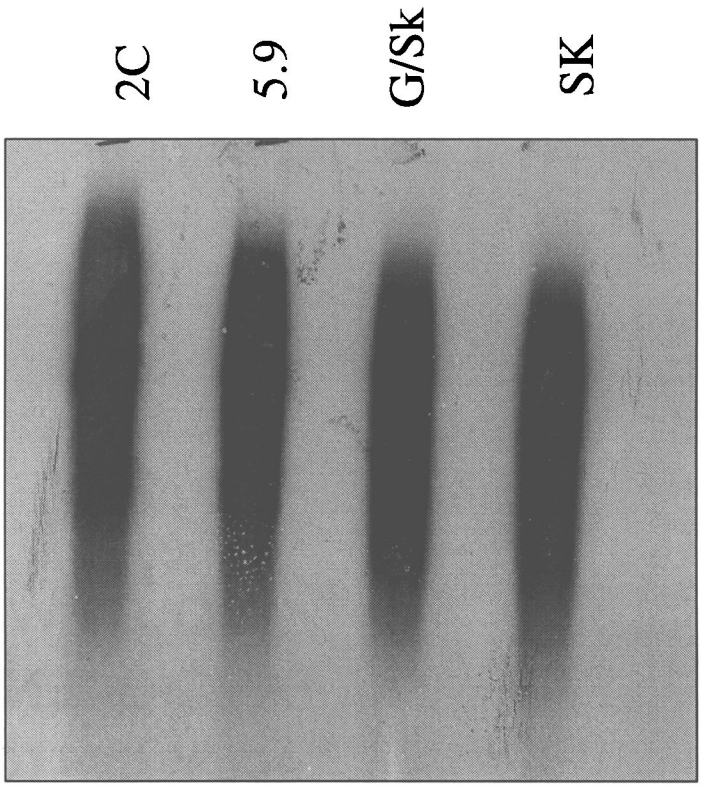


Figure 5. Efficiency test of the synthesis of double strand cDNA. First and second strand cDNAs of the tester, driver, and controls were synthesized as described in material and method. To confirm the efficiency of their synthesis, a test gel was run, dried on a 3MM sheet and wrapped in a

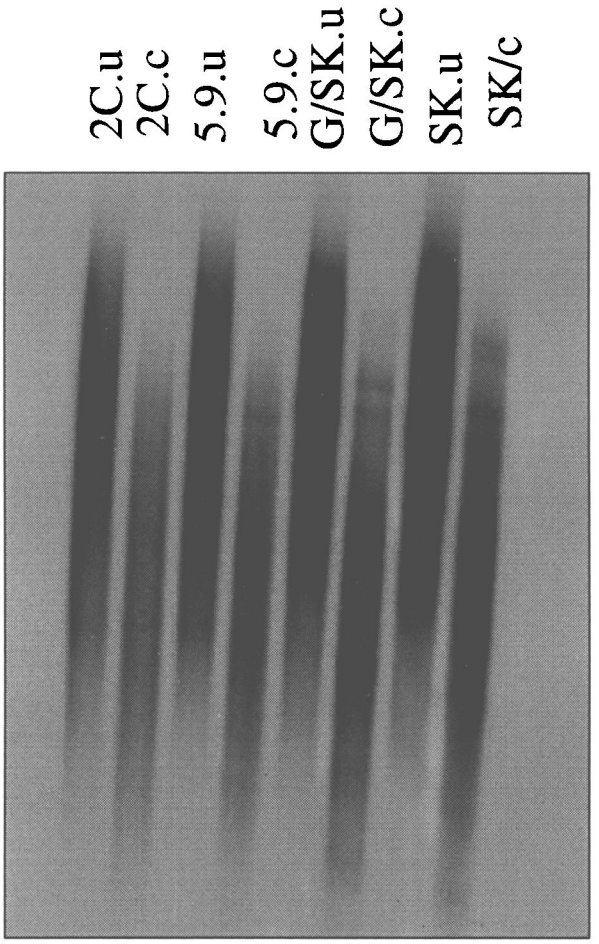


Figure 6. Analysis of RSA I digestion.

Uncut, ds cDNA and Rsa I-digested cDNA were run on a 1% agarose/EtBr gel. The results side by side show that after the Rsa I digestion, the average cDNA size becomes smaller.

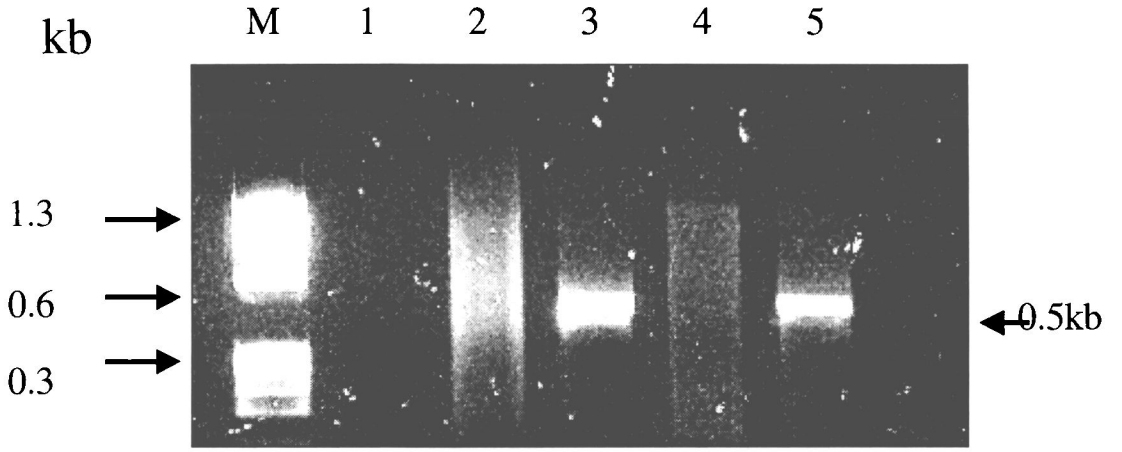


Figure 7. Ligation efficiency analysis.

RsaI tester digested cDNA fragments were subdivided into two samples and each sample was ligated at its 5'end with different double strand adaptor cDNA. To confirm the efficiency of the ligation reaction, PCR reactions were performed using PCR primer 1, specific to the two adaptors and 3' or 5' GAPDH specific primers. Lane M: ϕX molecular weight marker. Lane 1: negative control with specific primers but without tester templates. Lane 2: tester-adaptor 1 cDNA amplified using specific primer 1 and 3'GAPDH. Lane3: tester-adaptor1amplified using 3'and 5'GAPDH primers. Lane 4: tester-adaptor2R amplified using primer1 and 5'GAPDH primer. Lane 5: tester-adaptor2R amplified using 3' and 5' GAPDH primers.

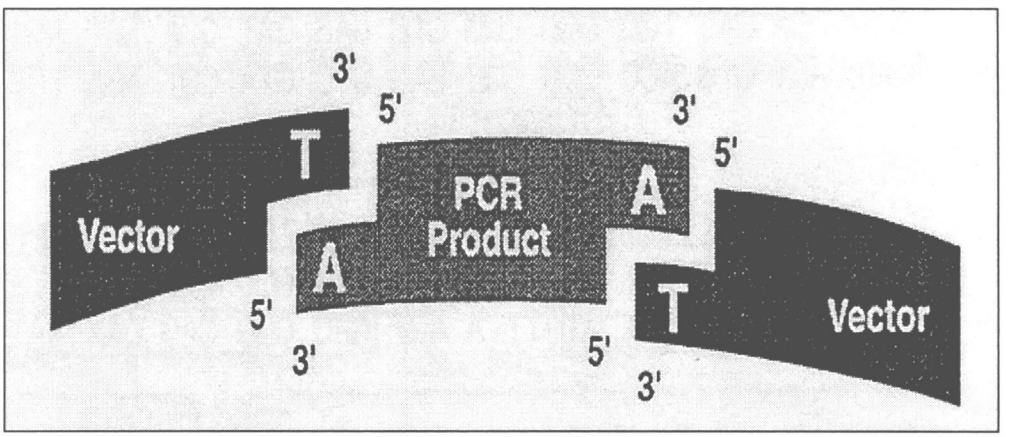


Figure 8. Concepts behind the TA cloning method. Taq polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3'ends Of PCR products. The linearized pCR II vector has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

IV. 3 Isolation and characterization of differentially expressed cDNA clones

To isolate and characterize differentially expressed cDNA clones, colonies were randomly picked from the subtracted library, and each bacterial colony was grown overnight in LB-amp in a 96 well plate. Amplification of each insert was performed directly on bacterial colonies, using nested PCR primer I and 2R. Figure 9 shows the resulting PCR products when resolved on a 2% agarose gel electrophoresis. All the PCR products were run on three different agarose gels. On gel I, ϕX molecular weight marker is on lane 1, the PCR negative control without template is on lane 2 and on the remaining lanes are the amplified inserts. On gels II and III, the molecular weight marker is also loaded on lane 1 and the amplified cDNA inserts can be seen on the remaining lanes. To screen the differentially expressed cDNAs, the resulting PCR products were first denatured and arrayed in duplicate on nylon membrane. Two identical blots were made. To use as probes for differentially screening, excess of adaptors was removed from the forward and reverse subtracted cDNAs by restriction enzyme digestion. The resulting digested forward and reverse subtracted cDNA products were ^{32}P-radiolabelled and hybridized with each cDNA arrayed on membrane. Positive hybridizations were observed with the forward subtracted cDNA probes as indicate arrows on figure 10, but no hybridization was observed with the reverse subtracted cDNA probe (data not shown).

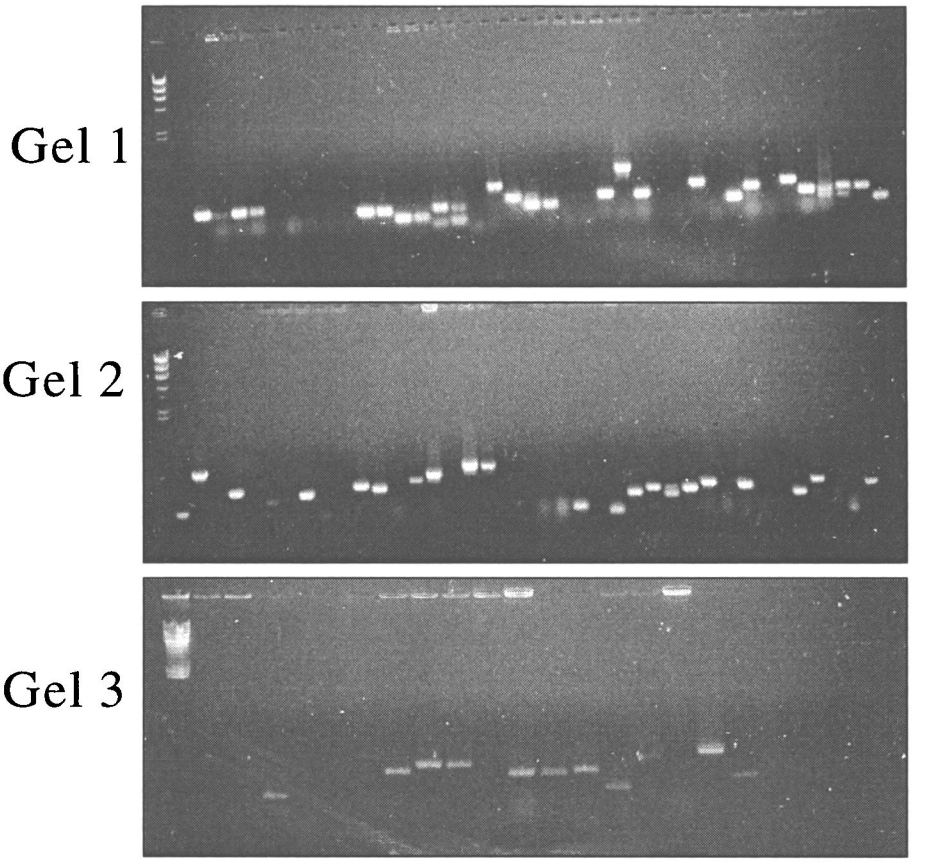


Figure 9. Amplification of differentially expressed cDNAs by PCR. Bacterial colonies were randomly picked from the subtractive library plate and grown at 37 °C overnight in 2 x YT-amp medium in a standard 96 well culture plate. Insert was amplified using each bacterial colony as template source, and nested PCR primers1 and 2R. The amplified PCR products were run on 1% agarose gel.

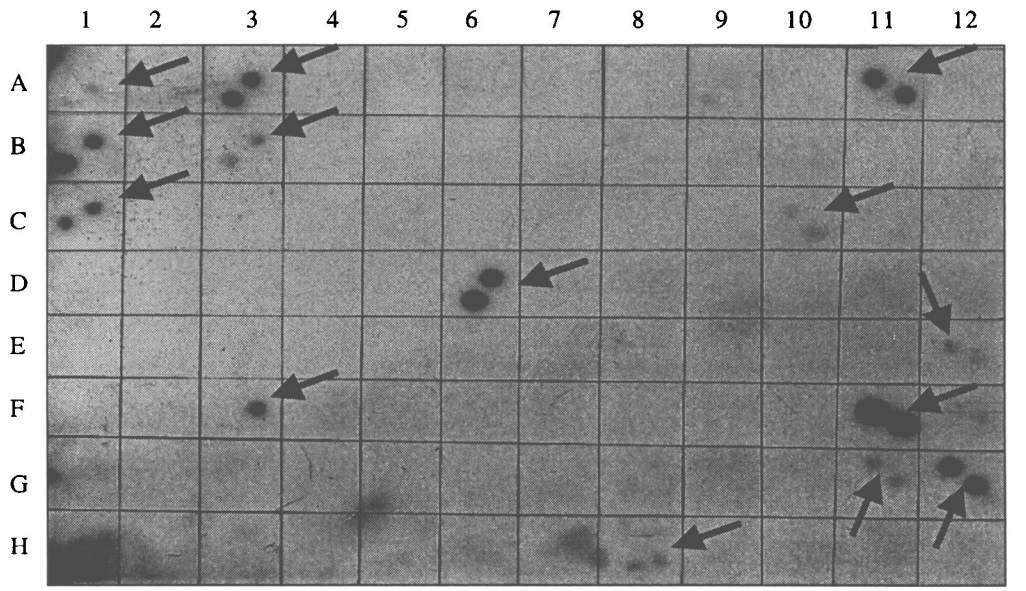


Figure 10. Dot-blot screening of differentially expressed cDNA clones. Amplification enriched PCR product from each clone was denatured and dot-blotted on nylon membrane. The array was hybridized to labeled subtracted cDNA after removal of the flanking adaptor sequences by *Rsa*I digestion. Positive clones are arrowed. Numbers on the rows and letters on the columns represent the 96 well plate equivalent replica on the filter membrane. DNA was spotted in duplicate.

To confirm the results of the differentially screened cDNAs, virtual northern hybridization experiment was carried out by blotting side by side the forward-and reverse-subtracted cDNAs on nylon membrane. Probes were prepared first by growing for plasmids purification, each bacterial clone harboring differentially hybridizing cDNA sequence. Differentially expressed cDNA insert, isolated by PCR using nested PCR primers 1 and 2R was P^{32}-labeled and hybridized with the virtual northern blot. As observed on Figure 11, all isolated and radiolabed differentially expressed cDNA inserts hybridized with the forward subtracted cDNA on the virtual northern. No hybridization was detected with the reverse subtracted cDNA. This result suggests that the differential screening experiment was very successful. All differentially expressed cDNA clones in addition to some clones randomly picked in the library were sequenced and database search was performed using the blast x program of the National Center for Biotechnology Information (NCBI). Table II shows the result of the data base search. The partial sequences of 3F and 1B cDNA clones are presented in figure 12. Database search of these two cDNA clones as indicated in figure 13 shows very limited identity to known proteins.

To determine cDNAs specifically expressed, northern hybridization was carried out using each cDNA as radiolabeled probe on total RNA extracted from various types of cell lines. The result of the northern blot revealed that 7 cDNA clones were expressed on all the cell lines except cDNA clones 1, 3F, 1B and 6D that are expressed only on 2C and A20 cell lines. Figure 14 shows the expression of 3F and 1B cDNA on total RNA of various types of cell lines. Ethidium bromide staining of the RNA gel is shown on the lower panel to demonstrate the equal amount of total RNA loaded. A complete

correlation was found between the expression of these two clones and the transcription from mouse genome. This finding was accomplished by southern blot analysis in which genomic DNA isolated from mouse spleen tissue was digested with PstI, EcoRI, or RsaI, the digests were electrophoresed in duplicate set and blotted on nylon membrane. Each set of enzymatic digest blot was hybridized with 3F or 1B cDNA probe. In figure 15, each probe displayed a different detection pattern of hybridization, suggesting that each probe is transcribed from a different gene in the mouse genome, although the possibility that the two cDNA clones could be derived from the same transcriptional unit via alternative splicing could not be ruled out.

Figure 11. Representative confirmation result of the differentially expressed cDNA clones by virtual northern blot analysis. A series of forward and reverse-subtracted cDNA pairs were electrophoresed on agarose gel and transferred onto nylon membrane filters. Cut filter strips containing forward and reverse-subtracted libraries were hybridized to selected labeled purified inserts derived from each positive clone identified by dot-blot screening array. F: forward-subtracted cDNA library; R: reverse-subtracted cDNA library.

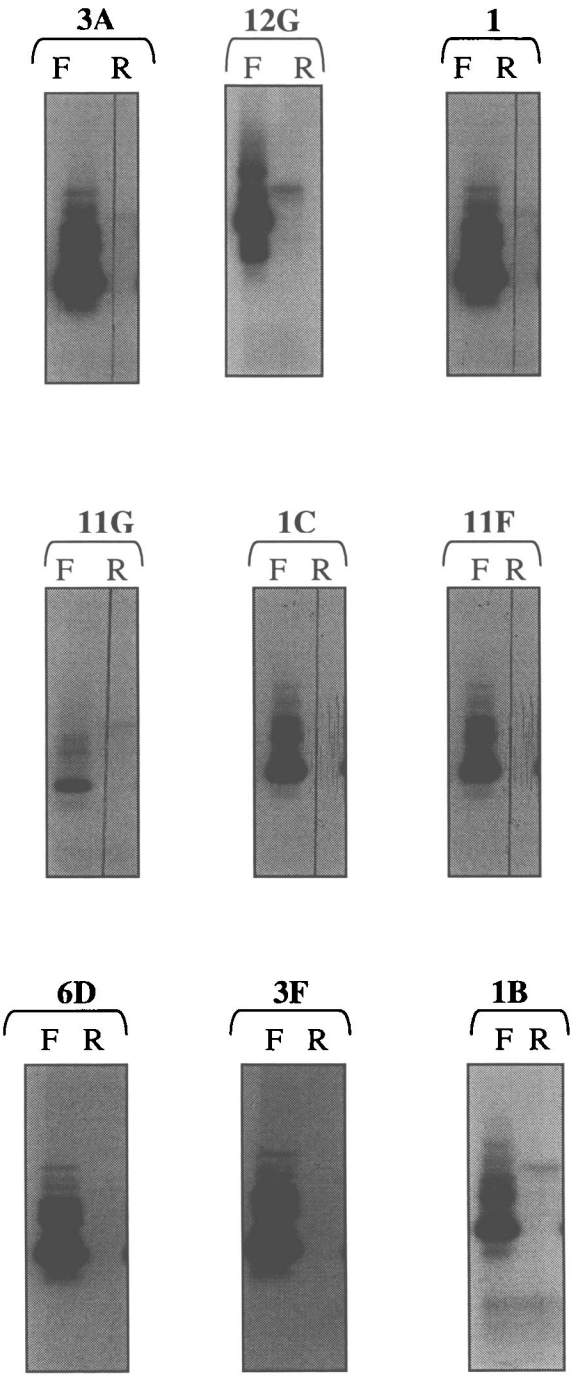

3A
F R
12G
F R
1
F R
11G
F R
1C
F R
11F
F R
6D
F R
3F
F R
1B
F R

Table 2. Preliminary protein similarities determined with the BLAST X sequence analysis program.

Clone #	Description	E value	Identities
7	Cerebellar postnatal development protein-1 (Lanp-like protein) (Riken cDNA 2810018A15gene)	$2e^{-50}$	96% / 101 aa
9	Glucocorticoid receptor (GR)	8.4	42% / 24 aa
12	Neuronal-specific septin 3	0.006	50% / 28 aa
1B	Early growth response protein 1 (EGR-1) (KROX-24 protein) (ZIF 268)	0.25	41% / 37 aa
10C	Probable ATP-dependent RNA helicase DDX20 (Dead-box protein 20) (Dead-box protein DP 103) (Component of GEMS 3) (Regulator of steroidogenic factor-1 (ROSF-1)	$1e^{-13}$	37% / 98 aa
3F	Transcription factor MTF-1 (MRE-binding transcription factor)	0.66	44% / 34 aa
12G	Hepatocyte growth factor-like protein precursor (Macrophage stimulatory protein) (MSP)	0.97	41% / 32 aa

3F

ACCAGAGCTGAGTTCTCAAAAGTTACAAGAAAGTTCAGTTAAAGATTAACAG
TTAAAGATTAAGGCTGAATAATACTGGGACAGGGGCCAAATATCGGTGGTCA
AGCACCTGGGCCCCGGCTCAGGGCCAAGAACAGATGGCTCTCAGACGTCATG
TTAGCAGAACTAGCTTCACTGATTTAGAAAAATAGAGGTGCACAGTGCTCTG
GCCACTCCTTGAACCTGTGTGTCTGCCAATGTTCTGACCAGATATGTGCCCAT
TGCTGAACCTTCATTAGACTCTTCCT**GT**

1B

ACCCGGGCGACGCATCTATCGGAAGACTGGCGCGCCGAGTGTGGAGTTTTTA
CCCTTTTTATAGGGCTGGGGCTGGGGAGCAAAAAGCGCGGTTACAGAAGCGA
GAAGCGAGCTGGTTGGTTAGTTCGAATAAGGCTTGGGGTATTTCTCGGTCATT
TGGGGAAACTGGGGTGGGACTTTTTAGTTCTAAACAATGTCTATTTTTAAGAA
ATGGGTATGGGAGGG**GT**

Figure 12. Nucleotide sequences of 3F and 1B cDNA clones. PCR products were cloned into pCR® 2.1 vectors and sequenced with SP6 or T7 primers using either Dye or BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI 377 automated DNA sequencer (ABI prism) according to the manufacturer recommendations. The underlined parts represent *Rsa*I ½-sites.

A/

```
Alignment between:
MTF1_MOUSE  TRANSCRIPTION FACTOR MTF-1 (MRE-BINDING TRANSCRIPTION FACTOR
and (Pasted_No.1.22:189-88)
        Length = 675
        Score = 27.8, bits (60.0), Expect = 0.66
        Identities = 15/34 (44%),    Positives = 20/34, (59%)

Query: 189      LLTXTSESHLFLALSRGPGA*PPIFGPCPSIIQP 88
                 LL  T+ S    A S GPG+ P  FG  P+++QP
Sbjct: 433      LLPATAPSAPPPAPSLGPGSQPAAFGSPPALLQP 466
```

B/

```
Alignment between:
EGR1_MOUSE  EARLY GROWTH RESPONSE PROTEIN 1 (EGR-1) (KROX-24 PROTEIN) (Z
and (Pasted_No.1.14:177-73)
        Length = 533
        Score = 28.2, bits (61.0), Expect = 0.25
        Identities = 15/37 (41%),    Positives = 21/37, (57%)

Query: 177      VPPQFPQMTEKFPKP--YSN*PTSSLLASVTALFAPQ 73
                 VPP FP     FP     S+  TS+ L+ +TA F+P+
Sbjct: 492      VPPAFPTQVSSFPSAGVSSSFSTSTGLSDMTATFSPR 528
```

Figure 13. Amino acid sequence alignment of A/ 3F and mouse transcription factor MTF-1, B/ 1B and mouse early growth response protein-1 (EGR-1). Identical and conserved (+) amino acid are indicated between the query and the subject. The alignment was made by using the BLAST X program (NCBI).

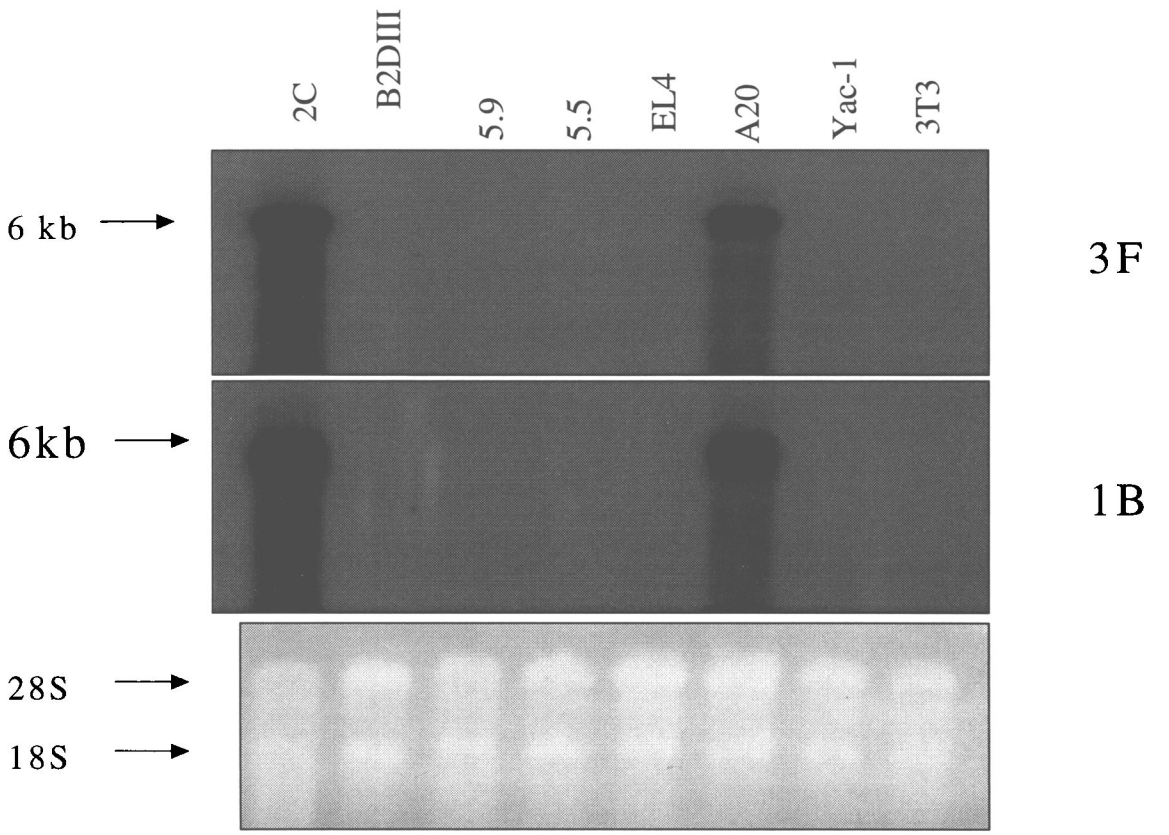


Figure 14. Northern blot analysis of 3F and 1 B mRNA expressions in various cell types. 2C, B2DIII, 5.9, 5.5, EL4, A20, Yac-1, and NIH-3T3 cell lines were grown as previously described. Total RNA was isolated from each cell line, electrophoresed on a formaldehyde denaturing gel, blotted, and hybridized with 3F and 1B cDNA probes. Each lane contains 10 µg of total RNA as measured by absorbance at 260 nm. Ethidium bromide stained of the RNA gel is shown on the lower panel.

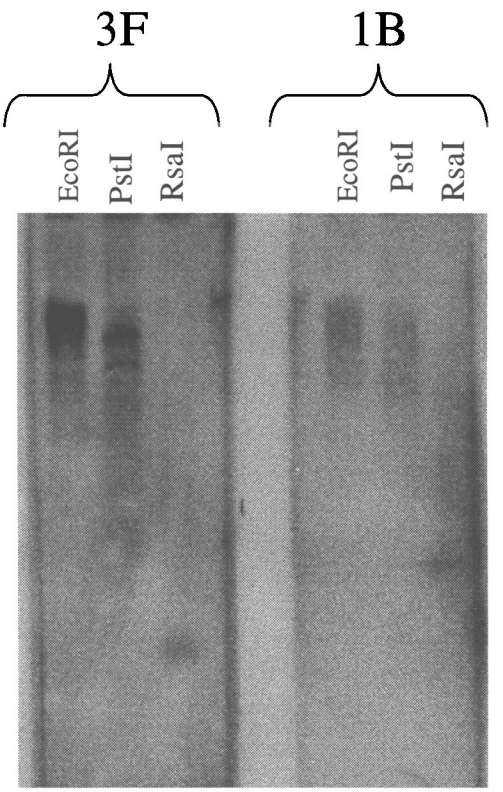
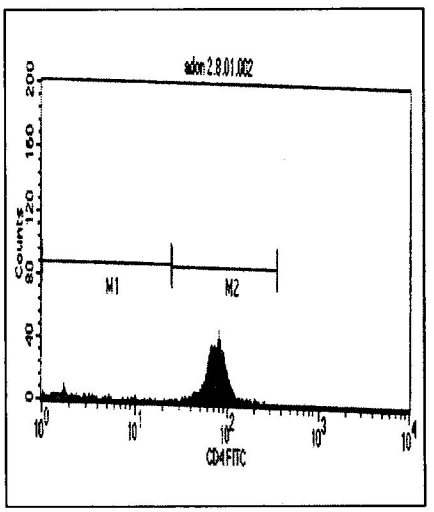


Figure 15. Southern blot analysis of genomic DNA. High molecular weigh DNA isolated from mouse spleen tissue after overnight digestion with EcoRI, PstI, or RsaI was fractionated by agarose gel electrophoresis, transferred to nylon membrane, and probed with 3F and 1B cDNA clones.

IV.4 Expression of 1B and 3F cDNA clones on $CD8^+$ and $CD4^+$ cytotoxic T cells purified from primary MLRs.

CD8 and CD4 cytotoxic T cells were purified from primary MLRs by negative selection using the stempSep separation method (see material and method section). The separated cells were labeled and then analyzed by flow cytometry and respectively 93% and 94.5% of pure CD4 and CD8 CTLs were obtained as indicates figure 16. Total RNA was immediately extracted from each purified cell, followed by reverse transcription of the RNA using oligo (dT) or random primers. Each reverse transcribed reaction was quantitated by a set of ribosomal 18 S genes specific primers. The expression of 3F and 1 B cDNA clones were analyzed by RT-PCR using a set of gene specific primers, and the same amount of each reverse transcription reaction used during the quantitation by the ribosomal 18 S primers. In figure 17, the 18 S primers show the integrity of the RNA and also the quantitation of the RT-PCR reaction. 3F and 1B cDNA clones are both expressed in CD8 cells derived sample RNA but not in CD4. This result suggests that 3F and 1B are cDNAs constitutively expressed genes in cells.

A)



B)

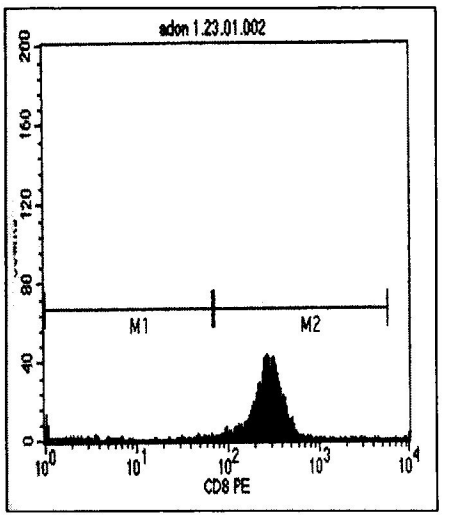


Figure 16. Flowcytometry analysis of cells purified from primary MLRs.

A/ Purified $CD4^+$ T cells stained with anti-mouse CD4.

B/ Purified CD8+ T cells stained with R-PE anti-mouse CD8a. The labeled cells were analyzed by flowcytometry and respectively 93 % and 94.5 % pure $CD4^+$ and $CD8^+$ T cells were obtained.

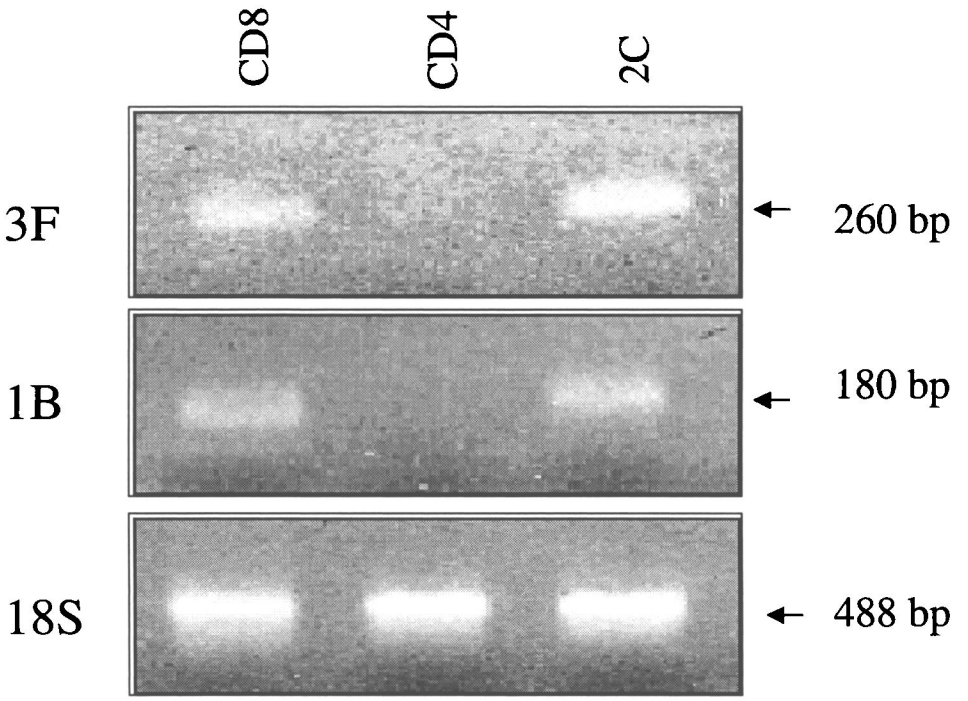


Figure 17. Analysis of the expression by RT-PCR of the 3F and 1B cDNA clones in cells purified from primary MLRs. PCR with a set of 18s rRNA primer was used to insure the RNA integrity.

IV.5 Initial cloning of 1B and 3F cDNAs

The 300 base-pair (bp) 1B and 3F cDNA fragments isolated through SSH were used as probes to screen a XbaI- $(dT)_{15}$ [an oligo (dT) primer including an XbaI restriction site]-primed mouse spleen 5'-STRETCH cDNA library λTriplEx (Clontech Laboratories, Inc., Palo Alto, CA). After three rounds of plaque purification, nineteen and thirteen positive individual phages were respectively isolated for 3F and 1 B cDNA clones as described in material and method. Each individual phage was converted to pTriplEx plasmid as described in material and method and the purified plasmids were run on agarose gel for the selection of plasmid with longer insert (figure 18A & B). To determine the size of each insert, all the selected purified plasmids were digested with EcoRI/XbaI restriction enzymes and the resulting restriction enzyme digestion products were electrophoresed on agarose gel (figure 19A & B). Only 1,554 bp of clone 14-1B were sequenced as shown by figure 20. The 1B cDNA fragment used as probe for the screening has not been detected yet. Database search still using the BLAST X program of the 1,554 bp shows very limited homology (33% / 51 aa) to mouse neuronal-specific septin 3 protein.

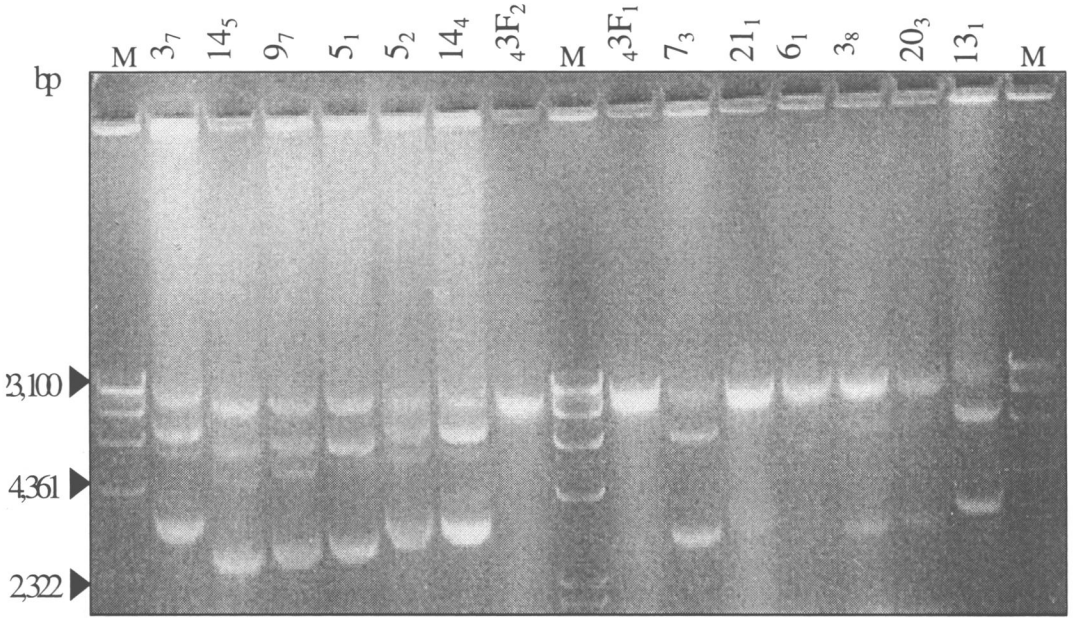


Figure 18-A. Agarose gel electrophoresis of pTrip1Ex plasmids.

After three round of plaque purification using 1B cDNA radiolabeled probe, thirteen positive phages were isolated as described in materials and methods. Each individual phage was converted to pTrip1EX plasmid (see materials and methods). The pTrip1EX plasmids with different insert sizes were run on a 1% agarose gel electrophoresis and photographed.

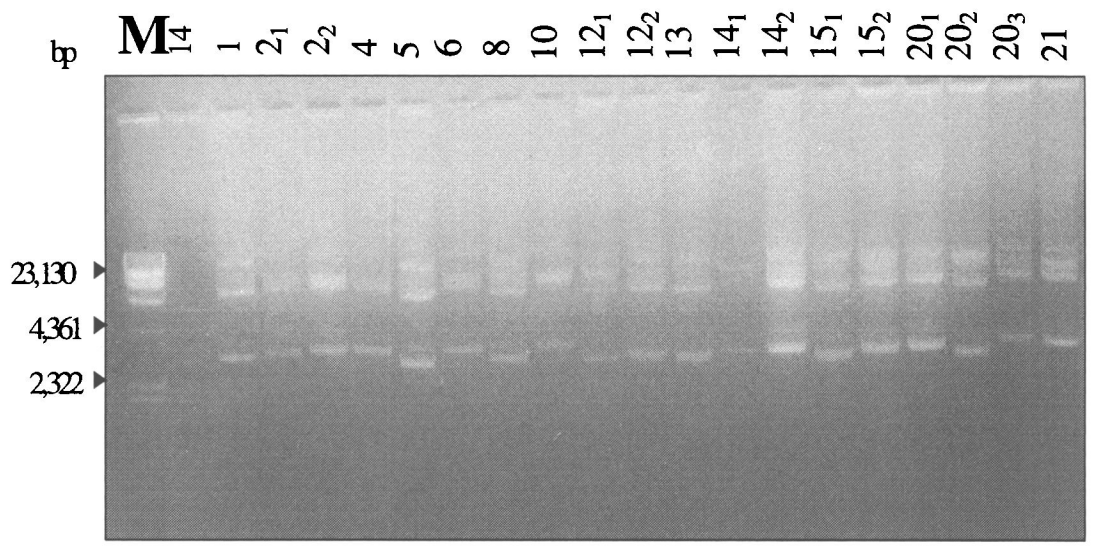


Figure 18-B. Agarose gel electrophoresis of pTriplEX plasmids.

After three rounds of plaque purification using 3F cDNA radiolabeled probe, nineteen positive phages were isolated as described in materials and methods. Each individual phage was converted to pTriplEX plasmid (see materials and methods). The pTriplEX plasmids with different insert sizes were run on a 1% agarose gel electrophoresis and photographed.

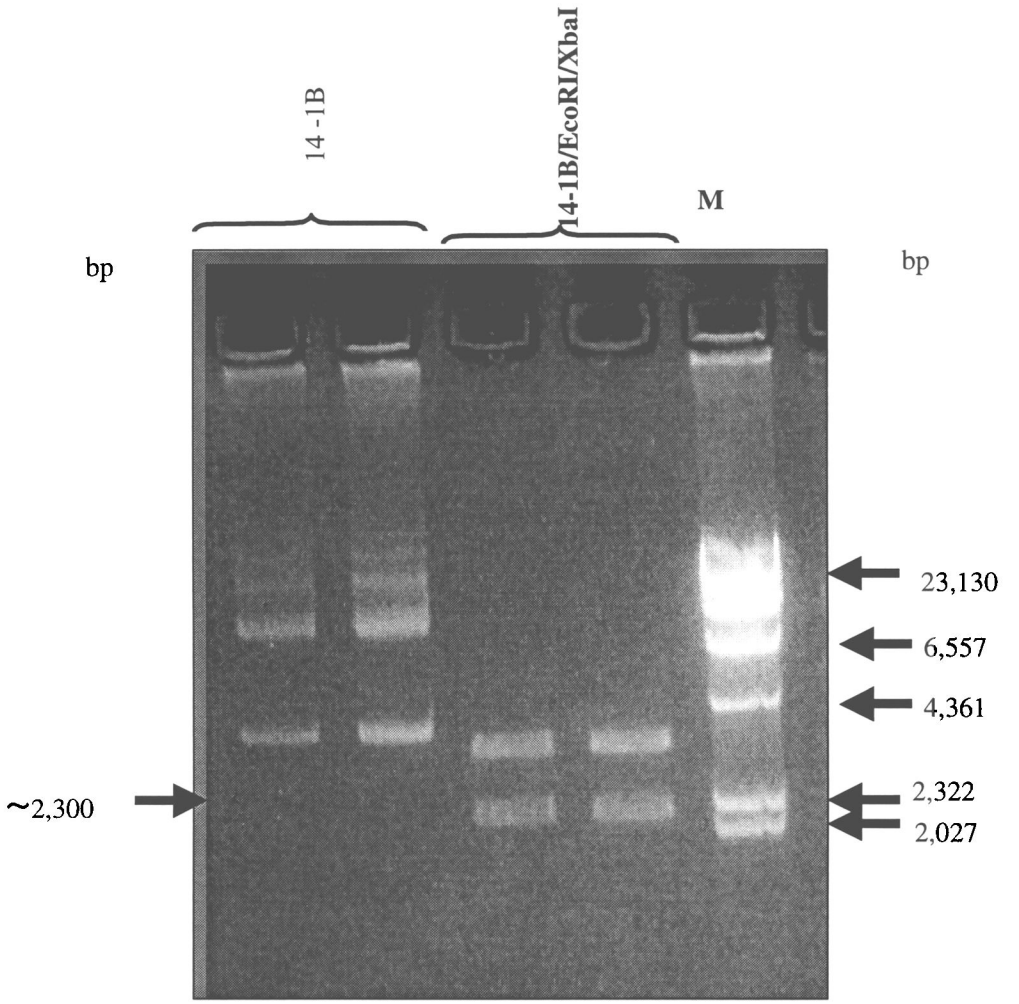


Figure19-A. Size determination of insert by restriction enzyme digestion.

The purified pTrriplEX plasmid (14-1B) was EcoRI/XbaI digested and run in duplicate on a 1% agarose gel. The non digested plasmid, also run in duplicate was used as control. As the photograph shows, 14-1B is ~2, 300bp in size. M represent λ HindIII molecular weight marker.

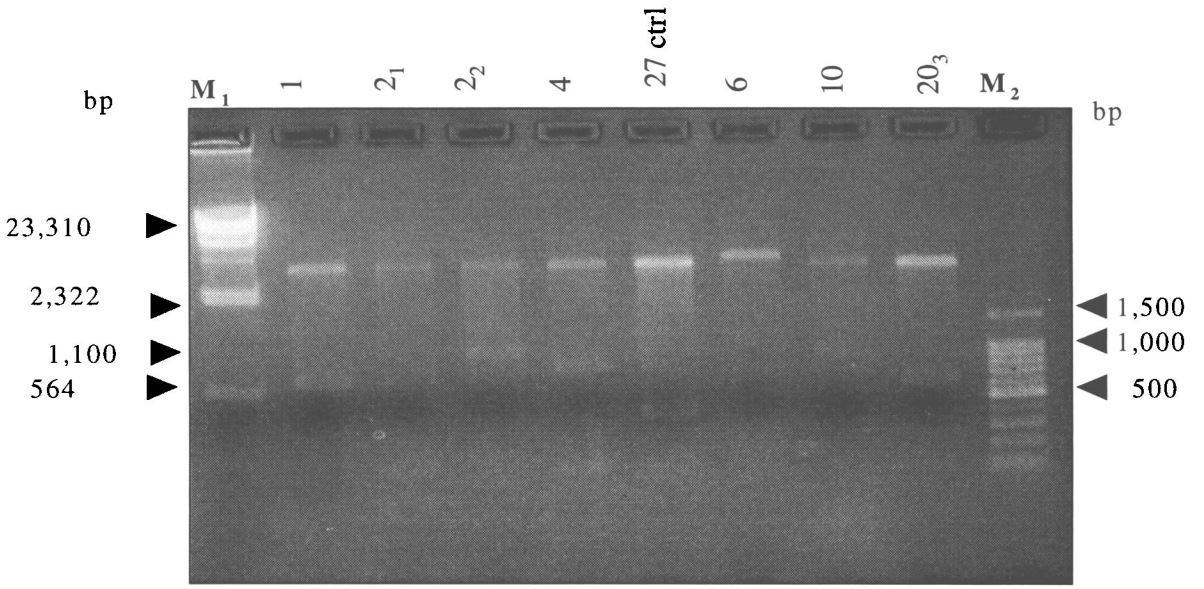


Figure 19-B. Size determination of insert by restriction enzyme digestion. The selected pTriplEX plasmids were EcoRI/XbaI digested and run on agarose gel electrophoresis. As the photograph indicates, clone 2_2 has an insert of ~1,100bp. M1 and M2 respectively represent λ HindIII and 100bp ladder molecular weight markers.

5' GAA TTC GCG GCC GCG TGC ACC GCG CCC GGT CTC CTC CCC GCG AGC GCG GAG CGG
CGA GCC CGG CGC GGC CAT GGG GAG TGG GAT GAG CCA GAC CCT GCC GGG CCT GTA
CAT TGG CAA CTT CAA AGA CGC AAG AGA TGC AGA ACA GTT GAG CAG GAA CAA GGT
GAC ACA CAT TCT TTC TGT GCA CGA TAC TGC CAG GCC CAT GTT GGA AGG GAG TTA
AAT ACC TGT GTA TTC CAG CGG CAG ACA CAC CAT CTC AAA ACC TGA CAA GAC ATT
TCA AAG AAA GCA TTA ATT CAT TCA TGA GTG CCG ACT CCA TGG TAG AGC TGT CTT GTA
CAT TGC CTG GTG GGG TCT CCA GGA GTG TGA CAT TGG TGA TCG CAT ACA TCA TGA
CTG TCA CCG ACT TTG GCT GGG AAG ATG CCT TGC ACC ACT GTT CGT GCG GGG AGG
TCC TGT GCC AAC CCC AAC CTG GGC TTT CAA AGG CAG CTG CAG GAG TTT GAG AAA
CAT GAA GTG CAC CAG TAT CGG CAA TGG CTG AGA GAA GAG TAT GGA GAG AAC CCT
TTG CGG GAT GCA GAA GAA GCC AAA AAT TAT TCT GGG TAA ATA TAA AGA GCA AGG
GCG CAT GGG AGC CCC GGC CTG GCA GCA GGC GGT GGA GCA GCT TGT GAC CCT GCC
TCC TCT CAC CTA CAA TAA CTA CAC AAC AGA GAC CTA ACA GAG AGA GCT GGT GTC
TGC CTT CCT GCT GCG GGT CTT CTG GGT TGC CTA CCA TGT GCT GGT GTG CCT GGT GTC
CTG GCT CCT GCC TCG CAG GAC TAC GAG AGG AGG TCG CAG CAA GGT GGA GCA CTC
AGG GCT CCT TCT CAG AAT ACC GCC CTA CTC AGG CTT TTC ACT CTC CCA TCT TCG CCC
CAT CTT TTC CTC ACC TGA ACT TGC CCA GCC TGG GAT GCT GCC CGG CCA CCG TGT ACT
TCT CGT ATG CGT GCA GGC GTG TGG ATG TGC ATG TAT GTG TCT AAG AGT GTG CAT ATA
TAC CTA CAA ATG TAT GCA TTG TGA ACA AGT ACA CAT GTA AAT GTG TCT CTG CAT GTG
GGC ACT GAG TGT TTA TGG TGC TGA AAG TTA TAA ACA TCC GCT GCC AGA ACT GCA
ATG GTC ACA TTG TTC AGT CCA CAT GGA AGT CAT TGA ACT TGG CCT CTG GAA AGC TAC
TCA CCA AGT AAC AGC TTA TGC CTG TGC TGA GTG AGA GCT CAG GGT GTG GGC AGC
TGG AAA CAG TGG TGT TCC AGA TTC TGA GAT GGC ACA GAG GAA GGG ACA GGA CCC
TCC TGA GGA AGA GTG GCA TAA TCC TAG AGA GTT TTA TGT CTG TGG GAA CAA GGG
AGG GGC TTT CTG AGC ACT GTC TTG GAC TTG ATA AGT ATA CTT GCC AGC CCG TCA TGG
CCC TGA GTT CCA CTG GTG CCT GCT CTG CGT GGG ACC AGC GTC ATT TGA CTT TCA TGG
TGA TAT GGT ATG GTG ACA GGG TGG ACC TGA GAC TCA GTA GCC CTA TAC CAG AGG
TCT GGC CCA CCT CTG TCT GCT TTT AAA CAC TTT AGC CTC TGG CTT AGC CTC TTG TTG
CAG GGG TCT CAT-3'

Figure 20. Nucleotide sequence of 14-1B clone.

The selected clone 14-1B of ~2,300 bp of obtained after the λ TriplEx cDNA library screening using probe 1B fragment. Only 1,554 bp was sequenced.

CHAPTER V
DISCUSSION

The major aim of this research was to isolate cDNA clones encoding proteins that may be involved in the passive resistance of cytotoxic T cells to granule-mediated lysis. As a source of the isolated cDNAs, the 2C cell line (a CD8 T cell) was used in subtractive hybridizations against an inflammatory CD4 T cell line, the 5.9 cell line. These two cell lines are known to be resistant to granule-mediated lysis, but after treatment with ATP depleting reagents (azide, cyanide, 2-deoxyglucose) and the 2C cell line remained strikingly resistant to granule-mediated lysis, while the 5.9 cell line became susceptible. Based on this observation, it was found that cell could be resistant to lysis either by ATP-dependent (active resistance) or ATP-independent (passive resistance) manner. The ATP-dependent mechanism may reflect the clearing of cell membranes of inserted perforin, like the mechanism used by many nucleated cells to reduce their susceptibility to lysis by activated complement, possibly via endocytosis of affected plasma membrane (63). This attribute could be generalized to all murine CD8+ clones. Verret et al. found that the active resistance mechanism appears to be well developed in $CD4^+$ T cells (both cytolytic and noncytolytic). The mechanism responsible for the ATP-independent (passive) resistance of $CD8^+$ CTLs is still unknown.

A subtractive cDNA library was constructed to identify cDNAs encoding proteins differentially expressed by cytotoxic T cells. Numerous cDNA subtraction methods have

been reported. In general, they involve hybridization of cDNA from one population (tester) to excess of mRNA or cDNA from other population (driver) and then separation of the unhybridized target fraction from the hybridized common sequences. Usually hydoxylapatite chromatography 68, avidin-biotin binding (69, 70, 71), or oligo $(dT)_{30}$-latex beads are used for the separation step (72). These methods had been successfully used for the isolation of important genes such as the T-cell receptor, but they are usually inefficient for obtaining low abundance transcripts. These techniques also require at least 20 μg of poly(A^+) RNA, involve multiple or repeated subtraction steps, and are labor intensive.

A recently PCR-based technique, the representational difference analysis (RDA), has been successfully used to enrich genomic fragments that differ in size or representation (73) and to clone differentially expressed cDNAs (74), but still does not resolve the problem of the wide differences in abundance of individual mRNA species. As a consequence, multiple rounds of subtraction are still needed (74). The mRNA differentially display (75) and RNA fingerprinting by arbitrary primed PCR (76) are faster methods to identify differentially expressed genes. However, both techniques have high level of false positives. Recently, protocols using cDNA microarrays with fluorescent labeled probes have been introduced (73, 77). The chip technology is highly efficient, but requires expensive equipment for making the microarrays and for detecting the fluorescence signals. Thus, chips technology is not widely available.

During this study, a new PCR-based cDNA subtraction method, termed suppression subtractive hybridization (SSH) was used. SSH selectively amplifies target cDNA fragments (differentially expressed) and simultaneously suppresses nontarget

DNA amplification. This method is based on the suppression PCR effect: suppression occurs when complementary sequences are present on each end of a single strand cDNA. During each primer-annealing step, the hybridization kinetics strongly favor (over annealing of the shorter primers) the formation of a pan-like secondary structure that prevents primer annealing. When occasionally a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-like structure (77). The subtraction described here overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics. The SSH eliminates any intermediate steps for physical separation of ss and ds cDNAs, and requires only one subtractive hybridization round. During the first hybridization, the excess of driver cDNA would have eliminated most of the common sequences between the tester and driver cDNA samples. However, quantitatively different cDNA species may still remain in the tester populations. To further eliminate common sequences, another excess of denatured driver cDNA was added to the samples in the second hybridization step, thereby further subtracting quantitatively different but common sequences between the tester and driver populations.

Digestion of the cDNAs with Rsa I restriction generated the largest average size of ~ 600 bp fragments. Although this may be a disadvantage when full length cDNAs are desired, dividing each cDNA into small fragments has two important advantages. First, long DNA fragments may form complex networks that prevent the formation of appropriate hybrids. Second, cutting the cDNAs into small fragments provides better representation of individual genes. Derived from related but distinct members of the

gene families, cDNAs often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (78). Further, different fragments from the same cDNA may vary considerably in terms of hybridization and amplification characteristics and may not hybridize or be amplified (79, 80). Thus, some fragments from differentially expressed cDNAs may be eliminated during the SSH procedure. However, other fragment from the same cDNA may be enriched and isolated. Once a small cDNA fragment is cloned and sequenced, numerous approaches, including several PCR based methods, can be used to quickly obtain corresponding full-length cDNAs (77).

The high level of enrichment, low background, and normalized abundance of cDNAs in the subtracted mixture make the SSH the method ideal for rapid cloning of cDNAs of differentially expressed genes. The feasibility of using the uncloned subtracted cDNA mixture as a hybridization probe makes this technique versatile and powerful.

The subtracted cDNA pool was analyzed after cloning in the pCR II cloning vector. Bacterial colonies randomly picked (106) from the library were directly amplified with nested PCR primer 1 and 2R. The PCR analysis revealed inserts in the range of 200-700 bp. Initially 96 cDNA clones were selected and tested for selectivity by reverse northern dot-blot hybridization with ^{32}P-labelled tester or driver cDNA. Only 13 differentially expressed cDNA clones were observed with the labeled cDNA tester and no hybridization was observed with the labeled driver (data not shown). To ensure exclusion of false positive clones, the dot-blot analysis was confirmed by hybridizing a blot of forward (F) and reverse (R) subtracted cDNAs run side by side with each selected

^{32}P-labeled differentially expressed cDNA. These differentially expressed cDNA clones in addition to 10 randomly picked from the library were sequenced. Pair-wise comparison using the blast sequence 2 programs revealed that 7 cDNAs are unique and clone 7 is identical to 8H, 1 B is identical to 1, and 3 F is identical to 6D. Of the 23 sequences, only 6 were redundant, suggesting that the subtracted cDNAs are normalized and highly complex. Northern analysis of the selected cDNA clones with total RNA extracted from 2C, 5.9 and others cell lines reduced the number of differentially expressed cDNA clones to two, the 1B and 3F cDNA clones expressed only in 2C but not in 5.9 cell lines. Cloned 2C and 5.9 cell lines, maintained in culture for several years with the support of high levels of IL-2, and repeated exposure to granules when periodically stimulated with targets may have induced the synthesis of proteins. The expression of 1B and 3F cDNA clones in purified cells from primary mixed lymphocytes reactions (MLRs) that had been in culture for only 5 days shows that these two cDNAs are expressed in $CD8^+$ CTL but no expression was observed in $CD4^+$ T cell. This result suggests that 1B and 3F are constitutively expressed cDNAs. Data base search of all selected cDNAs using the blast X sequence program gives the results presented in table II. BLAST X program was used for its ability to translate nucleotide sequences in all six reading frames and also to find short stretches of similarity to known proteins. None of the cDNAs sequenced were similar to known cytotoxic T cells' proteins such as perforin, $CD8^+$ T cell, $CD4^+$ T cell, or T cell receptor genes. Failure to identify these proteins may be due to the statistical limitations of picking only 106 clones from the constructed library, or these cDNAs may have been removed during the hybridization steps due to the similarities in their coding regions. Overall, the significance of the similarities presented

in table II exhibited limited proteins similarities with the BLAST X sequence analysis program, therefore these cDNAs can be considered as unknown and will require further sequence information, such as cloning of the full length to confirm their relationships to other genes. The results presented here show that 1B and 3F cDNA clones are expressed only in 2C, but not in 5.9 cell line and also for physiological relevance, these two cDNAs are expressed in $CD8^+$ T cell purified from primary MLRs, suggesting that they are not induced cDNAs due to the CTLs' culture conditions. Thus these two mRNAs are good candidates for functional evaluations that might demonstrate whether or not they specify protective proteins.

The first approach toward gaining clues regarding function was to obtain sequence information. Sequence data on 1B and 3 F reveals respectively limited identities to early growth response protein 1 (EGR-1) (KROX-24 protein) (ZIF 268) (41% / 37 aa) and transcription factor MTF-1 (MRE-binding transcription factor) (44% / 34 aa). Metallothionein transcription factor-1 (MTF-1) is a member of the Cys2His2 family of zinc finger transcription factors (81) that depends on the activation of both constitutive and stress-inducible expression of metallothioneins (MTs). MTs have well-established roles in metal homeostasis and in the detoxification of heavy metals. Moreover, they also confer protection against reactive oxygen intermediates, electrophilic anti-neoplastic agents, various mutagens, ionizing radiation, and nitric oxide (82, 83). Other studies indicate roles for the MTs in regulation of cellular proliferation and apoptosis (84-87), perhaps through an interaction of MTs with nuclear-κB-DNA complexes (88). MTF-1 may be also important for tumorigenesis not only through MT expression but also through its regulation of other genes. For example, MTF-1 activity is

necessary for the expression of γ-glutamylcysteine synthetase (89), the rate-limiting enzyme for the synthesis glutathione (GSH) (90). GSH is a major contributor to cellular defenses against environmental alkylating agents and oxidants, including reactive oxygen species generated by ionizing radiation (90). Thus, MTF-1 may be critical for modulating gene expression associated with malignant phenotypes such as resistance therapy.

The early growth response protein-1 (EGR-1), referred to as NGFI-A (91), Krox-24 (92) and zif/268 (93) is an immediate early gene, with zinc-finger DNA-binding properties that acts as a nuclear coupler of early cytoplasmic events to long-term alterations in gene expression (94). The Egr-1 protein binds to a specific GC-rich sequence in the promoter region of many target genes to regulate their expression (95, 96). It was found that Egr-1 has been functionally implicated in cell proliferation, differentiation and apoptosis during the differentiation of rat calvarial preosteoblasts (97). Even in some cells, Egr-1 not only acts as a transcriptional activator, but also as a repressor (98, 99) and has been shown to negatively regulate tumor growth in human fibrosarcoma cells (100). The relationship of 1B and 3F respectively to Egr-1 (41% / 37 aa) and MTF-1 (44% / 34 aa) is very limited in term of similarity, but the information provided above might be important clues related to their functions. Presently, we are searching for the full length of these two differentially expressed cDNA clones by screening premade cDNA libraries using 1B and 3F as radiolabeled probes.

Convincing functional demonstration may be difficult, even if 1B and 3F are in fact truly protection gene products. This may be so, as many phenomenon are occurring in resistant cells (regulation of gene expression, clearing of cell membrane, active ion

pumps that prevent or restore the marked changes in intracellular ion concentrations), any one of which may lead to cell resistance. However, one or both of these mRNAs might specify proteins that are essential in protecting cell, as is apparently the case of the products ced-9 gene, similar to mammalian Bcl-2, which acts to prevent apoptosis in mammals (101). In this regard, the MTF-1 and EGR-1 proteins encoded respectively by 3F and 1B cDNA clones might function as essential regulatory components. If this so, use of complementary oligonucleotides might favor programmed cell death. Such oligonucleotides are readily taken up by mammalian cells in culture, leading to hybridization-based blockage of translation or degradation of specific mRNAs. Transfection of full-length cDNA clones is another option that might lead to functional testing via regulated expression in other types of cells.

The chances of identifying a gene product that is essential in resistance of cells would be increased if more cDNA clones can be picked from the library. Our subtracted cDNA library contains many clones that did not hybridize with the subtracted cDNA probe in the differential screening. Some of these clones represent low-abundance mRNAs that might function specifically in the resistance of cells. Therefore, the library will be screened again using more sensitive detection procedures.

The SSH coupled with the PCR-Select differential screening procedure used to isolate 1B, 3F, and several other clones has several desirable features that contribute to its efficiency and reproducibility. The procedure requires only 0.5-2 μg of poly A^+ RNA and does not requires physical separation of ss and ds molecules (SSH manual, page 4). Furthermore, suppression PCR prevents undesirable amplification while enrichment of target molecules proceeds. Another useful feature is the PCR amplification step.

Because oligo (dT) primer-adaptor was used to prime synthesis, only cDNA inserts were amplified, thus increasing the purity of molecules to be used in construction of the enriched library. The amplification from this step also improves the chances of cloning sequences representing rare copy mRNAs, particularly in cases where transformation efficiency is low. Finally, inserting the secondary PCR products into pCRII using the T/A cloning kit (*INVITROGEN*), as opposed to directional insertion is a helpful feature in that it allows the cloning of cDNA fragments without internal enzyme-digestion.

In this study, the SSH coupled with PCR-Select differential screening was employed to enrich for cDNA clones that may be involved in the passive resistance of cells to granule-mediated lysis. From the one hundred six colonies randomly picked, only 13 clones were differentially expressed. Sequencing and northern analysis of the 13 clones reduced the number of differentially expressed genes to two, the 1B and 3F cDNA clones, that have no similarities to known proteins. These two cDNAs are probably involved in the passive resistance because they are isolated from a library constructed with mRNA from 2C cell and 5.9 cell lines. Further experiments such as cloning of the full-length and functional characterization of these two mRNAs need to be done to know their exact functions in T cells.

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